

# STUDIES IN IMMUNITY

BY

PROFESSOR PAUL EHRLICH

PRIVY COUNCILOR AND DIRECTOR OF THE ROYAL INSTITUTE FOR EXPERIMENTAL THERAPY,  
FRANKFURT, GERMANY

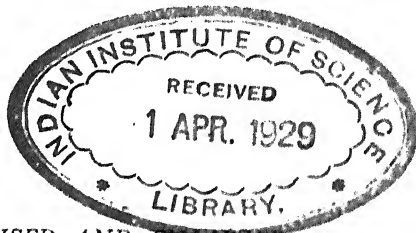
AND HIS COLLABORATORS

COLLECTED AND TRANSLATED

BY

DR. CHARLES BOLDUAN

BACTERIOLOGIST, RESEARCH LABORATORY, DEPARTMENT OF HEALTH, CITY OF NEW YORK



SECOND EDITION, REVISED AND ENLARGED

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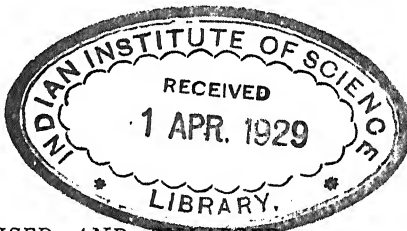
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To .

DR. ALTHOFF

PRIVY COUNCILOR, DIRECTOR IN THE PRUSSIAN MINISTRY OF EDUCATION, BERLIN, ETC.

THE ABLE FRIEND AND PROMOTER OF MEDICAL SCIENCE

THIS VOLUME IS DEDICATED IN

GRATEFUL APPRECIATION

## TRANSLATOR'S PREFACE TO THE FIRST EDITION.

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No apology is needed for presenting this translation of Ehrlich's classic studies in immunity, for a thorough knowledge of the master's work is indispensable to all workers in this field.

Attention is called to the fact that the important work done since the publication of the German edition has been included by the addition of three chapters, two by Ehrlich and Sachs and one, written expressly for this translation, by Prof. Ehrlich. The subject is thus brought up to about March, 1906.

CHARLES BOLDUAN.

## PREFACE TO THE SECOND EDITION.

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THE exhaustion of the first edition of this work affords the translator the welcome opportunity to add, not only Professor Ehrlich's new studies, but also some of his earlier papers which the recent publication of Bordet's Studies on Immunity renders desirable.

The translator may be pardoned for a feeling of gratification at the cordial reception extended this book both by the medical press and the profession at large. The appreciation shown has made the arduous, and usually thankless, work of translation one of great pleasure.

An exhaustive index has been added to this edition and will, it is hoped, greatly enhance its value as a work of reference.

For kind permission to reproduce articles from their publications, thanks are due to Messrs. August Hirschwald, Georg Thieme, J. F. Lehmann, and Gustav Fischer.

CHARLES BOLDUAN.

NEW YORK, February 1, 1910.

## PREFACE TO THE GERMAN EDITION.

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THE present volume embraces the greater portion of the studies in immunity published during the past few years by myself and my collaborators. While the publication of these studies in a single volume meets the request of numerous workers in immunity, it is hoped that the collection will at the same time fulfill another purpose, namely, to show clearly that my theory of immunity rests on so broad an experimental basis that it is practically identical with a summary of generalizations derived from an enormous mass of experimental data.<sup>1</sup>

When Behring's great discovery of antitoxin opened new paths for the study of immunity it was at once clear that further progress could be attempted in two ways. The first of these, having practical therapeutic results in mind, consists in bending all efforts to the production of various individual curative sera. The other method consists in seeking a deeper insight into the nature of immunity phenomena, and discovering the general principles underlying the same, for these in turn will aid practical progress.

By pursuing the latter method it has been found that the immunity reaction is merely a repetition of certain processes of normal metabolism, and that what is apparently a wonderful adaptation to the purpose is nothing more than the ever-recurring manifestation of primeval wisdom inherent in the protoplasm. I have endeavored to establish this experimentally and to show that the bond between

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<sup>1</sup> With a view of giving the reader a better idea of the technique ordinarily employed, and thereby to facilitate his introduction to this subject, I have had my colleagues, Dr. Morgenroth and Prof. Neisser, present the result of their extensive technical experiences with hæmolytic and bacteriolytic test-tube experiments, in two special chapters. (Chapters XXIX and XXX.)

what are at first sight very dissimilar biological processes is really a conception of the simplest kind.

The toxic metabolic products of bacteria, the artificially produced bacteriolysins, hæmolysins, and cytotoxins, and the majority of the ferments, probably always produce their effects by the co-action of two active groups in the molecule. One of these effects the union with the substance to be acted upon, while the other really produces the characteristic effect.

It is not surprising, in view of the enormous multiplicity of the vital phenomena, that this simple principle exhibits the greatest variations in individual cases. Certainly this corresponds entirely to what we constantly observe in the domain of biology. The cell, for example, occurs as a type in every living form, from the lowest plant to the highest animal. In principle it is ever the same; in the details of its structure, however, it is of endless variety.

But even from such complex phenomena as are exhibited, for example, by the artificially produced hæmolysins, it is possible to develop the fundamental principles of my theory, and thereby give a harmonious uniform explanation of the manifold phenomena with their peculiar specific relations.

My theory has developed essentially on the basis of chemical conceptions. I have been more and more forcibly impressed with the idea that in a study of the fundamental biological phenomena, the significance of morphological structure is far less than the significance of the chemistry involved. It is obvious that in order to effect a given chemical process certain mechanical conditions must be fulfilled. In other words the production of any chemical action necessitates the presence and the suitable arrangement of apparatus. The essential feature, however, is neither apparatus nor form, but the constituents involved; for without changing the apparatus hundreds of different combinations can be effected according to the components employed. Similarly in biology I believe that the morphological arrangement of the organs and cells is not the essential feature, but that this is rather to be sought for in chemical differences of the constituents.

I am convinced that the influence exerted by my theory will extend far beyond the limits of pure immunity studies, and that it is of considerable significance for an appreciation of vital phenomena. Furthermore, I believe that the theory is of great value in studying certain phenomena which dominate all life, namely, intracellular

metabolism, especially its two main phases, anabolism and catabolism. It has been shown that the substances obtained by immunization are nothing but the tools of normal cell-life, tools which we can thus isolate from their place of production and subject to an individual examination. This at once opens new paths for approaching the study of vital phenomena, which embraces not only the physiology and pathology of metabolism, but also certain other physiological problems such as those of secretion, heredity, etc.

At the recent Congress for Hygiene and Demography (Brussels), in which the chief problems of immunity were discussed, it was seen that my theory is not yet accepted by all the workers in this subject, there being still a few opponents. This was to be expected. Certainly nothing is more desirable in all scientific problems than the expression of different opinions, for as a result of experimental studies they lead to a deeper insight into the subject in question. Hence it is largely the opposition of Bordet and other distinguished workers in the Pasteur Institute that has spurred us on in our experimental labors, and caused us to establish the amboceptor theory more firmly than ever.

On the other hand it is very annoying when such authors as Gruber, who have absolutely no personal experience in the main questions, wage a bitter war merely because they have made a few literary studies; it is the more exasperating since they seek to make up the deficiencies in their arguments by the intensity and personality of their attacks. Such authors are in no position to correctly orientate themselves in the mass of true and false observations that each day's literature brings forth.

It was a great pleasure, therefore, to see one of the founders of the doctrine of immunity, R. Pfeiffer, and that distinguished representative of Paltauf's Institute in Vienna, R. Kraus, express themselves in favor of my theory. They confessed they had both really opposed the theory from the start, and that the main purpose in devising their various experiments had been to show that it was untenable. Just these, however, had convinced them that the side-chain theory not only afforded the best explanation for their results, but had even enabled them to predict these results. The chief problems now under discussion are: (1) the constitution of active cytotoxic sub-

stances, whether or not they are made up of two parts possessing different functions; (2) the union of specific amboceptors with the complements; (3) the plurality of complements. I am convinced that the near future will furnish so many additional arguments for the correctness of my views that all of these questions, as well as numerous others, will be decided in my favor. And the decision, I believe, will not be merely in favor of my views in general, but will extend even to the details.

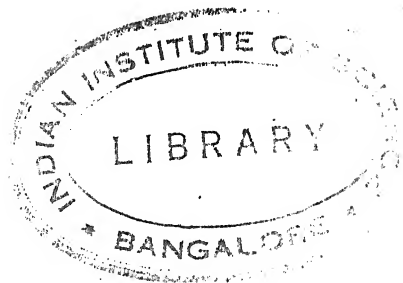
In a way, therefore, my position is like that of a chess-player who, even though his game is won, is forced by the obstinacy of his opponent to carry it on move by move until the final "mate."

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For the means to carry on these experiments, I am indebted first of all to the intelligent support which my scientific aims have received at the hands of my superiors, the Prussian Ministry of Education. I am especially grateful to the ministerial director, Dr. Althoff, who aided me in every way possible, and exerted himself to lighten my scientific labors. I may say that I was first spurred on to the immunity studies contained in "Die Werthbemessung des Diphtherieheilserums," and which have led to the formulation of the side-chain theory, by the remarks addressed to me by Dr. Althoff when the Institute was founded. It was he who begged that my first problem be an exhaustive study whereby the difficulties which had arisen in titrating and standardizing diphtheria antitoxin might be overcome. To this kind and able friend I have therefore dedicated this volume as a token of my gratitude and esteem.

PAUL EHRLICH.

FRANKFURT A. M., February 1904.



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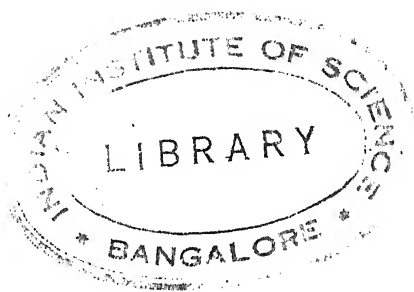
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## COLLECTED STUDIES IN IMMUNITY.

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### I. CONTRIBUTIONS TO THE THEORY OF LYSIN ACTION.<sup>1</sup>

By Prof. Dr. P. EHRLICH and Dr. J. MORGENROTH.

ONE of the most important advances in the study of immunity is the discovery of Pfeiffer's phenomenon, and it is to Pfeiffer's splendid observations that we owe the first and most important insight into the mode of action of the bacteriolytic immune sera.

As is well known, the phenomenon of bacteriolysis, first demonstrated by Pfeiffer in a guinea-pig immunized against cholera, consists in the immediate dissolution of cholera bacilli introduced into the abdominal cavity of the animal. The same takes place when the bacilli together with a small amount of immune serum are introduced into the abdominal cavity of a normal guinea-pig. Subsequently Metchnikoff (*Annal. Inst. Pasteur*, June 1895) showed that the phenomenon of bacteriolysis takes place also outside the animal body, in vitro, provided a small quantity of peritoneal exudate of a normal guinea-pig is added. Bordet (*Annal. Inst. Pasteur*, June 1895) was thereupon able to show that the immune serum is able to effect bacteriolysis in vitro without any addition, provided that it is absolutely fresh. On standing it becomes inactive; but it may be reactivated by even very small amounts of normal serum. Pfeiffer's ideas as to the nature of bacteriolysis were formulated by him in a very clever theory which he published in 1896 (*Deutsche med. Wo-*

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<sup>1</sup> Reprinted from *Berl. klin. Wochenschr.*, 1899, No. 1.

chenschr., 1896, Nos. 7 and 8) and which is here reproduced only in its main features.

The immunizing substances contained in cholera serum possess but feeble power to retard development. They are nothing but an antecedent form of substances developed in the peritoneum of the guinea-pig, specifically solvent for cholera vibrios. They are stored in the animal body in an inactive but stable form, somewhat as glycogen is stored in cell depots as an antecedent form of grape-sugar. When needed, these inactive substances of the serum can be converted into the specific active form through the active interference of the body-cells. This conversion can also be effected by the addition of a suitable serum. In this added serum a certain "something," present in very small amounts, effects the change, but is very soon used up in the process. In the animal body, on the other hand, this constituent is produced by the body-cells as long as the stimulus, caused by the presence of the cholera bacilli, lasts. The action of this substance is ferment-like. Bacteriolysis is also regarded as a ferment action, caused by ferments of a very peculiar kind. These ferments are fitted in an absolutely specific manner each to a single bacterial protoplasm, acting on this exactly as pepsin or trypsin acts on coagulated albumin. According to Pfeiffer, a somewhat distant analogy is seen in E. Fischer's yeast ferments, each of which can only split up a sugar of a definite composition. If this theory be correct, these specific ferments must exist in an active and an inactive modification.

Recently Bordet (*Annal. Inst. Pasteur*, Vol. 12, No. 10) published a series of experiments in which he showed that the laws which govern the specific bacteriolytic action of immune sera govern also certain specific solvent phenomena seen in red blood-cells.

Bordet treated guinea-pigs with repeated injections of defibrinated rabbit blood. The serum of animals so treated possesses the property of dissolving rabbit blood in vitro rapidly and with great intensity, whereas serum of normal guinea-pigs is unable to do this. Solution is preceded by a marked agglutination of the erythrocytes. On heating the specific serum for half an hour to 55° C. the hæmolytic power is destroyed, while the agglutinating power remains. The serum thus inactivated can again be rendered active by the addition of a certain amount of normal guinea-pig serum, and even of normal rabbit serum. The active guinea-pig serum has no effect on the red blood-cells of the guinea-pig itself or on those of pigeons, but

acts, though to a less degree, on the blood-cells of rats and mice. The active guinea-pig serum injected into the ear-vein of a rabbit is highly toxic to that animal.

The analogy existing between these phenomena and those of bacteriolysis is, as emphasized by Bordet, a very close one. This will be clear to the reader. Very likely, therefore, the mechanism of hæmolysis and that of bacteriolysis are very similar. The study of hæmolysis thus gains considerable theoretical significance.

Being so fortunate as to have at our disposal a considerable amount of appropriate serum, we have used this in order to gain a deeper insight into the nature of hæmolysis. This serum was derived from a goat which during eight months had been subcutaneously injected in somewhat irregular fashion with sheep serum rich in blood-corpuscles. The experiments were therefore made with sheep blood in the form of a 5% mixture of the defibrinated blood in 0.85% salt solution. By means of this great dilution certain sources of error arising from the constituents of the serum are avoided. These had manifested themselves in Bordet's experiments.

The serum of our goat rapidly dissolves sheep blood-cells *in vitro*. The degree of action of this serum can be accurately determined as follows: To each 5 cc. of the above-mentioned blood mixture decreasing amounts of the goat serum are added. It is then found that at 37° C. the specimens containing from 1.5 cc. to 0.8 cc. serum will become completely laky. After allowing all the specimens to act for two hours in a thermostat they are placed in a refrigerator and allowed to settle. It will then be found that there is a regular decrease in the amount of solution effected until finally the limit is reached in the specimen containing 0.1 cc. of serum. The serum of normal goats (we tried the sera of a number of different animals) is unable even in large amounts to dissolve sheep blood-cells. It is to be remarked that in the use of this immune serum in the amounts mentioned no clumping was ever observed to precede hæmolysis, although this phenomenon was carefully looked for.<sup>1</sup>

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<sup>1</sup> The serum of normal goats in doses of 1.5 cc. and over possesses the property to agglutinate sheep blood-cells, but this property seems to be subject to great individual and chronologic fluctuations. This agglutination of foreign bloods by certain normal sera, and which probably corresponds to the normal agglutinating action of sera on bacteria, was observed many years ago by Creite (*Z. f. rat. Med.*, Vol. 36) and later was again emphasized by Landois (*Die Transfusion des Blutes*, 1875).

If the immune serum is heated to  $56^{\circ}$  C., it completely loses solvent action. The addition of serum of normal animals : *inactivated* serum causes it to be *reactivated*. For this purpose can use not only normal goat serum but also normal sheep though the latter acts somewhat more feebly. This power of normal serum to reactivate an inactive immune serum is very easily lost. Even when the serum is kept on ice and protected against light it very soon shows a diminution of its reactivating power. In quantitative experiments, therefore, the inactive (stable) immune serum should always be reactivated by a perfectly fresh normal serum.

In hæmolysis, as in Pfeiffer's bacteriolysis, we are therefore to assume the existence of two substances. One of these, stable and quite resistant (stable), we shall call the *immune body*, following Pfeiffer's nomenclature. The other, normally present and highly labile (unstable), we shall for the present term *addiment*.

Although our results in the main agree with those of Bordet we must at once call attention to one difference in our observations. As already mentioned, the action of our goat serum on the blood-cells is *not* preceded by any agglutination. From this it follows that the agglutination cannot be considered a preparatory step necessary for the hæmolytic action, as Bordet seems to assume. The specific agglutinin has no relation whatever to the hæmolytic immune body. Similarly, according to the views of eminent bacteriologists, the specific bacteriolytic substances have no relation to the agglutinins. The lysins may exist independently of the agglutinins and these again independently of the bacteriolytic substances. The reader is reminded of the interesting observations of Pfeiffer and Kolle. These investigators described an immune serum which was strongly bacteriolytic but which did not at all agglutinate (Centralblatt f. Bakt., 1896, Vol. XX, Nos. 4 and 5). On the other hand E. Fränkel and Otto state that if a young dog be fed on typhoid cultures, the dog's serum will acquire agglutinating but not bacteriolytic properties. Similarly, if a frog is treated with typhoid bacilli the frog serum will agglutinate such bacilli. They remain in the lymph sac of the animal, however, not only alive but virulent. (Vidal and Sicard, Comptes rend. Soc. de Biol., XI. 27-97).

Pfeiffer's original theory sought only to explain in general the mode of action of the specific bacteriolytic substances. It did not concern itself with the questions how or where they originated. It was

order to throw some light on these problems that Ehrlich devised his side-chain theory.

At first Ehrlich's theory was applied to the origin of the anti-toxins and to the chemical relation existing between the toxins and certain atomic groups of the protoplasmic molecule. Pfeiffer himself applied the theory to the substances specifically bacteriolytic for cholera bacilli, and was able to demonstrate experimentally that the source of these bodies was in the spleen, the bone-marrow, and the lymph bodies (Pfeiffer and Marx, Zeitschr. f. Hyg., Vol. 37, 1898). Wassermann, who in his well-known tetanus experiments had furnished the first demonstration of the soundness of the side-chain theory, succeeded in showing the source of the specific typhoid bacteriolysin. The study of these bacteriolytic processes brought up a number of important questions directly concerning the side-chain theory, and we felt compelled to examine these experimentally.

According to Ehrlich's theory, if any substance, be it toxin, toxoid, ferment, or constituent of a bacterial cell or of a blood-corpuscle, possess the property of combining with side-chains of the protoplasm, the possibility is given for the formation of a corresponding antibody. The antibody, according to the theory, must possess such a group as will fit the haptophore (the specific combining) group of the invading substance. The soluble body, therefore, produced in response to the invading substance (toxin, toxoid, etc). must combine chemically with the latter. If the invading substance is in soluble form, as, for example, the toxins, the neutralization proceeds in the solution. If, however, it is not directly soluble, being originally an insoluble part of, say, a bacterial or blood cell, then the dissolved antibody in the blood will be abstracted from its solvent fluid and anchored by the cell particle. In the well-known experiment of Wassermann on tetanus poison, the same thing is seen. In this the invading substance (tetanus toxin) is abstracted from its solution and anchored by the crushed brain cells. In order to maintain the analogy we should expect that in our experiment *the immune body dissolved in the goat serum would be anchored by the erythrocytes of sheep blood.*

The manner of procedure in this experiment is very simple and consists in the addition to sheep blood, or a dilution of the same, of immune serum which has been heated to 56° C. in order to destroy its solvent properties. The mixture is then centrifuged to separate the cells and the fluid. In case the immune body has been anchored

by the blood-cells, the clear fluid should be free from prove this we have merely to add to some of this clear blood-cells, and a sufficient amount of addiment in the serum. If the fluid is free from immune body, the blood-cells remain undissolved. The centrifuged sediment must be tested for the presence of immune body. The sediment as much as possible from fluid, is mixed with salt solution and a sufficient amount of addiment. If a corresponding amount of immune body has been anchored by the blood-cells, they will remain undissolved. One of our numerous experiments follows:

4 cc. of a 5% mixture of sheep blood-cells are mixed with 1.3 cc. inactivated serum from our immunized goat. The mixture is allowed to stand for fifteen minutes at 40° C. and then carefully centrifuged. The supernatant clear fluid is poured off, mixed with 1.3 cc. of sheep's blood and then with 0.8 cc. serum from a normal goat. The mixture after being kept in a thermostat at 37° C. for two hours and then allowed to settle in the cold, shows no trace of immune body.

The centrifuged sediment, freed as much as possible from fluid by means of filter paper, is mixed with 4 cc. physiological saline solution and with 0.8 cc. normal goat serum. This mixture is kept for two hours in a thermostat at 37° C. and then centrifuged. The sediment is found to be completely dissolved or very nearly so.

In this experiment in which a sufficient amount of immune body was used, we see that complete union took place between immune body and the blood-cells, resulting in the entire abstraction of the immune body from the fluid. We have found that the same result is obtained at lower temperatures, even at 0° C. That this is a chemical union and not a mere absorption is seen by experiments with other bloods. Thus the red blood-cells of rabbits and of goats are not of affinity whatever for this immune body.

*As a result of these experiments, therefore, and in confirmation of the side-chain theory, we must assume that the immune body is anchored to the blood-cells by a specific haptophore group which anchors it to the blood-cells.*

The next important question was that concerning the effect of the addiment on the red blood-cell. This was studied in a series of experiments exactly similar to that of the previous experiment. In each experiment the mixture was mixed with addiment, the mixture centrifuged, and the results tested separately, by the addition of immune body to the mixture in the presence of addiment. We varied our experiments greatly.



as time and temperature conditions were concerned, but the result was always the same; *the red blood-cells did not combine with a trace of addiment. This is in direct contrast to their behavior toward the immune body.*

Having now determined the behavior of the blood-cells to immune body and addiment separately, it remained to see what the affinities of the blood-cells were when both of these bodies were present at the same time. The solution of this problem offers many technical difficulties. Practically it will be best to make the mixtures so that there will be just the proper amount of the two ingredients to effect complete solution of the blood-cells. We found that if we mixed 1.0 to 1.3 cc. of our inactivated goat serum with 0.5 cc. normal goat serum, this would just suffice to dissolve 5 cc. of a 5% mixture (in saline) of sheep blood-cells. If this mixture is placed in the thermostat, complete solution will ensue; but because an excess of the solvent substances has been avoided, the process does not take place rapidly. Usually it is completed at the end of  $1\frac{1}{2}$  to 2 hours.

If the mixture is kept at  $0^{\circ}$ - $3^{\circ}$  C., no solution occurs, and if it is then centrifuged and examined according to the methods just studied, the red blood-cells will be found to have loaded themselves with immune body, leaving the addiment in the fluid. The experiment shows that under the conditions mentioned, addiment and immune body exist in the fluid entirely independent of one another.

It still remained to determine the combining affinities at higher temperatures. A preliminary trial showed that if we used the proportions above mentioned and kept such mixtures in an Ostwald water-bath at  $40^{\circ}$  C. for six, ten, thirteen, and eighteen minutes respectively and then centrifuged, only in the first two tubes did the fluid remain colorless, while in the other tubes it was distinctly red. In the experiments at this temperature we therefore adopted a time limit of ten minutes. A tube of the above-mentioned mixture was allowed to remain in the water-bath at  $40^{\circ}$  C. for ten minutes and then centrifuged. The results were as follows:

The sediment mixed with salt solution shows hæmolysis of a moderate degree. (This occurs even if the sediment is mixed with ice-cold salt solution, centrifuged, and then again mixed with salt solution. By this manipulation the last trace of fluid originally adhering to the cells is removed.) Solution becomes complete when new addiment in the form of normal serum is added to the mixture. The centrifuged fluid does not, by itself, dissolve blood

added to it, or it does so in only a very limited degree. When, however, new immune body is added, the blood-cells are completely dissolved.

From these experiments we conclude that the sediment this time contained both components, though not in equivalent proportion, for there was an excess of immune body which became manifest only on the addition of new addiment. Corresponding to this the centrifuged fluid contained only faint traces of immune body and an excess of addiment.

The explanation of these phenomena presents no difficulties. It must be assumed that under certain circumstances the immune body and addiment enter into loose, readily dissociated chemical combination. This combination is hastened by heat and retarded by cold in entire conformity to the views previously expressed by Ehrlich (*Werthbemessung des Diphtherie-heilserums*, Jena, 1897). On the other hand, the affinity existing between blood-cells and immune body must be very strong, for these combine completely even in the cold. *We must therefore assume that the immune body possesses two different haptophore groups, one with a strong affinity for the corresponding haptophore group of the red blood-cell, and the other of feeble chemical affinity, which is able to combine more or less completely with the addiment present in the serum.* At 30° C., therefore, the red blood-cell attracts to itself not only the free molecules of immune body, but also those which have already combined with the addiment in the fluid. In the latter case the immune body represents in a measure a link which ties addiment to the red blood-cells and subjects these to the action of the addiment. In agreement with Pfeiffer, we regard the phenomena appearing under the influence of the addiment as analogous to digestion, and we shall probably not err if we regard the addiment as having the character of a digestive ferment. Morgenroth, by the experiments in which by immunization he successfully produced an antibody against rennin ferment, has made it very probable that the ferments, like the toxins, possess two groups, one a haptophore group and the other the actual carrier of the ferment action.

With this preliminary analysis all the various phenomena are now readily explained. We assume that the immune body combines with the small amount of digesting ferment normally present in the blood, and then, by means of its other haptophore group, fitting, for example, to red blood-cells or bacteria, carries this digestive

action over to these cells. From this we see also why the digestive action becomes manifest only on the addition of immune body. This brings the ferment, present in the serum fluid in such small quantity, to the blood-cells in comparatively large amounts, thus concentrating and increasing its action. It is possible and even probable that only a few substances with digestive properties exist in the blood, perhaps only one; but that a countless variety of specific immune bodies can exist there, as Gruber, among others, assumes. In that case we must assume that in these immune bodies there is always one group which fits only to the cells or substances used to excite its production, but that all these immune bodies possess an atomic group in common which effects the combination with the digestive substance. On this assumption it is very easy to explain by means of the side-chain theory the otherwise difficult problem of the mode of origin of the lysins. According to Ehrlich's definition, the side-chains possess definite atomic groups which are able to combine with certain other atomic groups and so increase the protoplasmic molecule. As far back as 1885 (Sauerstoff Bedürfniss des Organismus) Ehrlich had pointed out that the atomic groups thus anchored to the living substance were much more readily oxidized and that they therefore represent the nourishment (*κατ' ἐξοχήν*) of the cell. The study of immunity has considerably extended this view and taught us that the antibody represents such thrust-off side-chains; further, that the immunizing process consists in forcing the particular organism to produce these side-chains in surplus amount in conformity with Weigert's theory of cell injury. It is of course very probable that these side-chains, according to their special function, will be differently constituted. If a side-chain is designed to assimilate relatively simple substances, we may believe that the possession of a single combining group will suffice. Very likely the side-chains which anchor toxins are of this simple type. But it is entirely different when a giant molecule (albumin molecule) is to be assimilated. In this case the anchoring of the molecule is only a preliminary requisite. Such a giant molecule is useless to the cell and can only then be utilized when it is broken up by fermentative processes into smaller parts. It will be particularly advantageous to the cell if its "grasping arm" is at the same time a carrier of a fermentative group which can at once be brought to bear on the anchored molecule. We see such well-adapted contrivances (in which the grasping apparatus also possesses digesting properties) in a whole

series of higher plants. For example, the tentacles of *Drosera*, which may be regarded as grasping arms in the widest sense, secrete a strong digesting fluid.

If, then, we see that lysin action does not occur with toxins, but only when the contents of cells are absorbed, be these bacteria or blood-cells, we must conclude that in the latter case large-moleculed albuminous substances are concerned. These are much more complex in structure than the toxins, which represent mere cell secretions. For the assimilation of the highly complex bodies we therefore assume the existence of side-chains of a peculiar kind. These, besides their combining group, possess another group which by fixation with special ferments causes the digestion of the complex substances. If, by means of the immunizing process, one succeeds in having a surplus of these side-chains produced, they will be produced with both these functional groups and thrust off into the blood as immune body. This explains the wonderful contrivance whereby the injection of a bacterium is followed by the production of a substance which destroys this bacterium by dissolving it. This phenomenon is nothing but the reproduction of a process of normal cell life.

## II. CONCERNING HÆMOLYSINS.<sup>1</sup>

### SECOND COMMUNICATION.

By Professor Dr. P. EHRLICH and Dr. J. MORGENROTH.

IN a previous paper<sup>2</sup> we demonstrated the relations existing between the red blood-cells to be dissolved and the two components of a specific hæmolysin produced by immunization. It will be remembered that we termed the two components of the specific serum *immune body* and *addiment*. We were able to show that the immune body combines with the erythrocytes of the species whose blood was injected, since it has a specific affinity for these cells. We showed further that the addiment, the unstable (labile) ferment-like body which effects the solution of the blood-cells, is tied to these cells indirectly by means of the immune body.

Proof was thus afforded that, in conformity with the requirements of the side-chain theory, the immune body possesses one haptophore group by means of which it combines with the erythrocytes of the corresponding blood, and a second haptophore group with less affinity by which it combines with the addiment and transfers the action of the latter to the blood-cells.

At that time we availed ourselves of the serum of a goat which had been treated for some time with subcutaneous injections of a sheep serum rich in blood corpuscles. Corresponding to this treatment, the serum of the goat possessed a moderate degree of solvent action on sheep blood-cells.

In order to continue these studies it seemed essential to make use of a serum derived from an animal treated for some time with full blood, a serum that would accordingly possess a higher degree of activity. For this purpose we began the immunization (Nov. 12

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<sup>1</sup> Reprinted from Berl. klin. Wochenschr. 1899, No. 22.

<sup>2</sup> See pages 1-10 of this volume.

and Feb. 24) of two male goats by injecting them subcutaneously with increasing amounts of defibrinated sheep blood. In a short time a strongly active serum was produced in both animals, and we were able to observe how, following the general laws of immunization, its activity increased. The course of the immunization did not manifest any peculiarities. It should, however, be remarked that on the days following the injection of a considerable amount of blood (350 cc.) not the least decrease in the activity of the serum could be observed, in contrast to the experiences with tetanus or diphtheria immunization.

So far as the general method employed in the following experiments is concerned, it was the same as that mentioned in the first paper. The blood was always used in the form of a 5% suspension in physiological salt solution. At the time of these experiments the serum of buck I was able to dissolve the sheep blood completely in the proportion of 0.2-0.3 cc. serum to 5 cc. sheep blood mixture; 0.03-0.07 cc. serum were able to produce a just noticeable amount of solution. Of the serum of buck II, 0.15-0.2 cc. sufficed for complete solution. It should be mentioned that the serum of buck II even before immunization possessed a slight solvent effect on sheep blood. This, however, was so slight that 4.0 cc. of the serum were not nearly able to dissolve 5 cc. of the 5% blood mixture, and 1.2 cc. serum produced only a just noticeable amount of solution. Heating the serum to 57° C. for half an hour destroyed this action, as it did also that for rabbit and guinea-pig blood.<sup>1</sup>

With the sera of these two bucks we were now able to proceed with our experiments. The combination of the immune body with the erythrocytes of the sheep at 0° C. can be readily demonstrated, for at this temperature and by the employment of proper amounts of serum no solution takes place. The serum was allowed to act on the sheep blood for twenty-four hours, care being taken to keep the mixture at 0° C. The blood-cells were then separated by means

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<sup>1</sup> On examining the sera of a large number of normal goats one will find some sera which possess this feeble solvent power for sheep blood. Thus the normal goat sera which we employed for control tests in our first experiments, and which were used in great number, failed absolutely to show any solvent action, but at most manifested only a variable degree of agglutinating action. This will be seen from our reports at that time. In our first communication we had already called attention to the great variability of the agglutinating property.

of the centrifuge, and they showed by their behavior that they had combined with the immune body. They did not dissolve on the addition of physiological salt solution, but dissolved when addiment in the form of normal goat serum was added. In contrast to this, both components combined with the sheep blood-cells when the mixture was kept at room temperature (about 20° C.) even for only eight minutes. The blood-cells, separated by centrifuge and washed with physiological salt solution to free them from traces of serum, were mixed with more salt solution and placed in an incubator, where they dissolved in considerable quantity.

These new and stronger immune sera therefore exhibited properties in relation to the sheep blood-cells entirely analogous to those of the serum previously described by us. On the other hand in certain respects their behavior was entirely different.

The serum described by Bordet, as well as that of our goats,<sup>1</sup> lost its hæmolytic power when heated for half an hour to 56° C. This has been shown by Buchner to be true of all normal hæmolytic sera. *The sera of our two bucks even when heated for three-quarters of an hour to 56° C. showed only a scarcely appreciable diminution of their solvent action on sheep blood, while their normal solvent action on guinea-pig blood and rabbit blood was entirely destroyed.* Even when the serum was heated to 56° C. for three hours or when, after mixing with equal parts of water, it was heated for one and one-half hours to 65° C., it showed merely a reduction in its solvent action for sheep blood, but not a destruction of this action.

Our preliminary experiments on the combining relations had shown us that the action of these hæmolysins was due to the presence in the serum of a specific immune body and an addiment. It was therefore clear that we were here dealing with an addiment of a very peculiar kind, which was distinguished from the addiments of all hæmolysins heretofore known by its extraordinary resistance to thermic influences. This property must pertain to the addiment itself and cannot be ascribed to the presence of another substance in the serum increasing its resistance, for such a substance would have served to protect the hæmolytic bodies normally present.

In order, however, to analyze these phenomena completely, it was absolutely essential to obtain the two components of the complex

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<sup>1</sup> This refers to the female goats. The male goat is always designated "buck" by Ehrlich and Morgenroth. [Translator.]

serum, the immune body as well as the addiment, in a free state. In the ordinary specific hæmolytic serum the former is usually readily obtained because the addiment is destroyed by slight heating. In the case of our serum, however, heating proved ineffective, so it became necessary to adopt other means. Experience having taught us that the addiment is, as a rule, more readily destroyed than the immune body, we could expect to accomplish our purpose by using stronger destructive agents of a chemical nature. After a number of trials we have finally made use of the following procedure: One part of our serum is mixed with one-tenth part normal hydrochloric acid, the mixture digested at 37° C. for 30 to 45 minutes, and then neutralized. It will be found that the serum has then lost its solvent power for sheep blood-cells; but that it still possesses immune body in scarcely decreased amount can be shown by re-activating the serum.

The isolation of the immune body made it possible finally to demonstrate the combination of the immune body at higher temperatures, 20°-35° C. This combination is seen to be quantitative, i.e., *the sheep blood-cells are able to combine with all the immune body present in that quantity of serum which in its active state would just suffice for their complete solution.* For example, to 5 cc. of the 5% blood mixture, 0.15 cc. of the serum inactivated with hydrochloric acid is added, it having been previously ascertained that this amount of active serum just suffices for complete solution. The mixture is allowed to stand for half an hour at room temperature and is then centrifuged. To the sediment 2.0 cc. normal goat serum are added, and to the clear fluid some additional sheep blood mixture and 2.0 cc. normal goat serum. The sediment thus treated will be seen to dissolve completely, whereas the blood-cells added to the clear fluid remain intact despite the presence of the addiment. This shows that all the immune body combined with the sedimented sheep blood-cells.

The addiment necessary for this reactivation is present in *normal* goat serum, as can be seen from the experiment. This is true for all goat sera thus far examined by us, although the amount varies. It will be recalled that we had found the original addiment which fitted the immune body was able to withstand heat. The question therefore at once arises whether normal serum also contains such heat-resisting addiments. As a matter of fact this was found to be the case in a number of goats examined by us. When the serum of these goats was heated for  $\frac{1}{2}$  to  $\frac{3}{4}$  hr. to 56° C. and its normal hæmolytic



properties for other blood-cells were entirely destroyed, it was still able to typically reactivate the particular immune body here concerned.<sup>1</sup> In another series of goats, however, the result was different, for heating the serum to 56° C. destroyed its reactivating properties completely. These sera then contained exclusively a thermolabile addiment which, like the thermostabile addiment, fitted the immune body. We must therefore conclude that the immune body developed by this immunization is capable of being activated by addiments of two kinds, which differ from each other by their resistance to thermic influences and which are both present in normal serum.

It is probable that both kinds of addiment can be present in goat serum at the same time, but that in most cases only one, the thermolabile, is present. The varying behavior toward thermic influences, manifested by the sera of our immunized animals, would thus be easily explained. *We assume that the same immune body was present in both cases, but that the serum of the goat first immunized contained only the thermolabile addiment, while the sera of the animals examined later contained also the thermostabile addiment.* In this connection, the fact that, previous to the commencement of immunization, we were able to demonstrate a considerable content of thermostabile addiment in the serum of the third animal (buck II) is of considerable interest.

Having thus arrived at some understanding of the action of the hæmolytic sera produced by immunization it seemed essential that we extend our investigations to the *hæmolytic properties of normal sera*. These properties had long been known and had been studied particularly by Buchner and his pupils.<sup>2</sup>

The fact that the hæmolytic action of normal serum is destroyed by moderate heat led us to believe that the normal hæmolytins are

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<sup>1</sup> As it is thus possible to destroy all the normal lysins (which interfere with the experiment) it ought to be possible to determine whether a similar heat-resisting addiment also occurs in the serum of other species. We succeeded in demonstrating its presence in varying amounts in the serum of a sheep and of a calf, but failed to find it in serum of a dog or rabbit.

<sup>2</sup> It is very probable that certain forms of hæmoglobinuria originate through analogous hæmolytins. Many years ago Ehrlich showed that the hæmoglobinuria ex frigore was caused, not by any particular sensitiveness of the erythrocytes to cold, but by certain poisons produced, especially by the vessels, as a result of the cold. Possibly also such autolysins play an important rôle in the convalescence of severe anæmias.

not of simple constitution; but the experimental solution of this problem was attended with great difficulties.

The primary tests necessary to demonstrate the complex constitution of a lysin are very readily made on a number of series. They consist in this, that a serum which dissolves certain red blood-cells at ordinary temperatures is mixed with these cells at  $0^{\circ}$  and allowed to act at this temperature for some time. For example, goat serum is mixed with guinea-pig blood-cells, for which it is normally hæmolytic. The mixture is kept at  $0^{\circ}$  and then centrifuged. The clear fluid is mixed with an additional amount of blood-cells and tested in the usual manner for its hæmolytic power. In this way it was easily shown that through this procedure the serum had lost part of its power, but that this was completely restored by the addition of some of the same serum previously inactivated by heat. According to our previous experience these experiments show that this serum contains two substances: one, which we shall call *interbody*, possessing two haptophore groups and analogous to the immune body; the other, an *addiment*, which we shall hereafter term *complement*. Further, they show that of these two bodies the blood-cells combine preponderantly with the interbody. The decrease in the power of the serum is thus explained by a lack of interbody, and this is supplied by the addition of inactive serum.

In experiments of this kind we have succeeded with the following combinations: goat serum, sheep serum, calf serum, and dog serum, with guinea-pig blood.

Although the demonstration of the lack of interbody is extremely simple, the counter-demonstration, that this interbody has combined with the sedimented blood-cells, is extraordinarily difficult; for in this demonstration a completely isolated complement is essential. The production of a complement to fit the *specific* interbody obtained by heating the serum of our immunized goat is extremely easy, for it is found in all normal goat serum and can also be obtained from immune serum by means of elective absorption.

It will be well to analyze the conditions governing this elective absorption by means of which interbody and complement can be separated. Complete separation will be possible when, under the circumstances prevailing at the time, the affinity of the interbody's haptophore group for the blood-cells is greater than the affinity of its haptophore group for the complement. A measure of the

relative affinity is found in the degree of temperature at which combination occurs. In the case of the lysin obtained by immunization, which has already been described, the combination of the blood-cells with the corresponding haptophore group of the immune body took place at  $0^{\circ}\text{C}.$ ; the combination of the second haptophore group with the complement took place only at a higher temperature. At  $0^{\circ}\text{C}.$  the fluid would therefore contain immune body and complement in a free state, i.e. uncombined. In this case, of course, it is possible completely to abstract the immune body from this mixture by means of the red blood-cells. This is the most favorable case. Its direct opposite will be one in which the affinity of the two haptophore groups is exactly equal. In that case the blood-cells will invariably combine with interbody + addiment in such a manner that *equal* amounts of the two components are withdrawn from the fluid. Naturally between these two extremes all kinds of intermediate phases may exist showing variations in the degree of affinity of these two groups. It seems to us that the most frequent case is that in which the affinity of the hæmotropic group of the interbody is not much greater than that of the group fitting the addiment. In this case we are unable to produce free addiment by treating the mixture with erythrocytes; a certain amount of interbody always remains in the serum so that the latter does not completely lose its solvent property. Such sera, which still possess solvent property, cannot, of course, be used for experiments in activation.

In our investigations on normal sera we met with this last case surprisingly often, and it was this circumstance that made the study of the complements so difficult. We therefore sought to find another method of procedure, one by which these difficulties could be avoided.

For analytical purposes it is essential, as already stated, to have both components of the serum, viz., interbody and complement, in an isolated form. The interbody can at any time be obtained from the normal active serum by heating, but the production of the complement from the normal serum is not entirely successful because of the above-mentioned difficulties.

We therefore proceeded on the assumption that every blood serum may contain a whole series of different ferment-like bodies, among which some would be capable of assuming the rôle of complement. It was of course clear that such a combination of circumstances would only be a fortunate chance occurrence, and that only

by examining a large number of separate cases would such a favorable combination be found. As a matter of fact after a rather long search, we succeeded in finding such cases.

As is well known, dog serum dissolves guinea-pig blood with great energy. If it be heated to 57° C. it loses this power, in accordance with the usual rule. However if to the 5% guinea-pig blood mixture some of this inactive dog-serum is added, and also a sufficient quantity of normal guinea-pig serum (about 2 cc. to 5 cc. of the 5% blood mixture), complete solution takes place. This fact can be explained only by assuming that the guinea-pig serum contains a complement which happens to fit the haptophore group of the interbody derived from the dog, and that it thus reactivates this. In this case the proof is all the more convincing because solution is effected by the addition of serum of the same species from which the blood-cells are derived. This serum should be the best possible preservative for the cells, for it represents their physiological medium.<sup>1</sup>

By means of these experiments we regard it as positively proven that the hæmolytic action exhibited by a serum, normally or in response to immunizing procedures, is due, in the cases examined by us, to the combined action of *two* substances.

Now that we had at our command the interbody of the hæmolysin solvent for guinea-pig blood, derived from dog serum, as well as a complement which reactivated this, we were ready to proceed to the last of our demonstrations.

To each of two test-tubes containing 5 cc. 5% guinea-pig blood 0.2 cc. inactive dog serum were added, after it had previously been ascertained by experiment that 0.2 cc. dog serum previous to heating were just sufficient completely to dissolve this amount of guinea-pig blood. The mixtures were allowed to remain at 20° for half an

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<sup>1</sup> We succeeded also in finding other combinations in which an analogous relation in greater or less degree could be demonstrated. Of these we may mention: 1) guinea-pig blood, inactive calf serum, guinea-pig serum; 2) sheep blood, inactive rabbit serum, sheep serum; 3) goat blood, inactive rabbit serum, goat serum; 4) guinea-pig blood, inactive sheep serum, guinea-pig serum. The fact that such an interbody, i.e., one derived from one animal species, finds fitting complements not only in its own serum but also in that of different species, is of considerable importance in the question whether curative sera can be made harmless to man by means of pasteurization. Possibly this would serve to explain why heating of the diphtheria curative serum, introduced by Spronck, has not realized the expectations a priori held out for the procedure.

hour and then centrifuged. The sediments thus obtained were washed with salt solution and again centrifuged. If now to one of these sediments physiological salt solution was added, and to the other 1.5 cc. guinea-pig serum, complete solution resulted in the latter, while the former remained undissolved. This proves that the interbody was completely anchored by the blood-corpuscles. The fluid obtained by centrifuging did not dissolve guinea-pig blood, even when considerable guinea-pig serum was added. It did not, therefore, contain any free interbody derived from the dog serum first added.

By these experiments we became convinced that hæmolysis in general is due, not to a simple body, but to the combined action of two distinct substances. At the present time we have no general method to demonstrate this for each individual case, and the solution of the problem therefore is now possible only under either of the above-mentioned favorable conditions: (1) when the two haptophore groups of the interbody differ greatly in their affinity; and (2) when, by means of a combination whose discovery depends on chance, an activating complement is found. Where these conditions are not fulfilled, the solution of the problem, for the present at least, is impossible. This, for example, is the case with ichthyotoxin, the hæmolytic constituent of eel serum. It is extremely easy to inactivate this eel serum, slight warming for fifteen minutes to 54° C. sufficing, but thus far we have been entirely unsuccessful in reactivating it, because we have been unable to find the requisite complement.

Considering their multiplicity, it is but natural that we are only just getting a deeper insight into the nature of the substances in normal blood serum. It is obvious also that a great many questions whose solution is of importance present themselves, especially in connection with the substances discussed by us.

The first question to be considered is that of the multiplicity of the hæmolysins contained in a given normal serum. According to our observations it is very probable that the ability of serum of one species to dissolve the blood-cells of various other species is dependent on the action, not of a single lysin, but of several lysins. If, for example, dog serum dissolves the blood-cells of guinea-pigs and of rabbits, it must be assumed that a multiplicity of interbodies and of corresponding complements effects this action. Some of the ways in which the solution of this problem can be approached are as follows:

(1) The isolated destruction of single lysins by means of chemical influences.

(2) The binding of the different lysins by means of corresponding species of blood, thus making their elective removal possible from red blood-cells this procedure, to which we shall return in the subsequent article, offers many technical difficulties. On the other hand, with a different kind of specific constituent of the blood, namely, the agglutinins, this method is easily applied, as can be seen by the experiments of Bordet<sup>1</sup> made in connection with the experiments and carried out by the methods employed by us.

(3) A separation of the lysins also seems possible through immunization, by means of which one is able to obtain an antilysin against the normal lysins. Thus Kossel, Camus, and Gley, by immunizing animals with the strongly globulicidal eel serum, have obtained a serum which neutralizes the action of this eel serum, in other words, one containing an antilysin. Evidently this reactively formed antibody thrusts itself into the hæmotropic group of the interbody thus deflects this from the erythrocyte. Our attempts, based on these premises, to produce an *isolated* antibody for some of the lysins have thus far been unsuccessful. Thus a serum obtained from rabbits after these had been treated with goat serum, protected the rabbit erythrocytes against solution by goat serum. At the same time, however, it protected the blood of guinea-pigs and rats against the same influence, and even prevented the hæmolytic action of the eel serum on rabbit blood. From this fact we must conclude that immunization with *one* serum produces a whole series of antilynsins. Clearly this is to be explained by assuming that the eel serum contains a great number of different complexes possessing haptophore groups, of which many, whether they are toxic or not, are able to excite the production of corresponding antibodies.

This surprising multiplicity of substances, present in the blood which possess haptophore groups (hæmolysins, agglutinins, ferment, and antiferments) is very readily harmonized with Ehrlich's conception. According to his conception all these substances represent side-chains of the protoplasm, which have been thrust off and have entered the circulation. The physiological object of the side-chains is, as Ehrlich stated in 1885,<sup>2</sup> to bind assimilable substances to the protoplasm so that these may serve as nutriment for the

<sup>1</sup> Inst. Pasteur, March 1899.

<sup>2</sup> Ehrlich, Sauerstoffbedürfniss des Organismus. Berlin, 1885.

A large part of these side-chains may, under suitable circumstances, be thrust off and thus appear in the blood.

Considering the large number of organs in the body and the manifold chemistry of their protoplasm, it should not surprise us that the blood, which represents all the tissues, can be filled with innumerable side-chains; and it is not at all astonishing, considering the constantly changing chemistry of the organism (influenced by a large number of factors such as race, sex, nutrition, labor, secretion, conditions of the surrounding medium, etc.) that the serum should be subject to constant qualitative fluctuations. Such variations are seen in the examples already mentioned, showing the behavior of sera of normal animals. Goat serum at one time possesses a slight solvent action on sheep blood, at other times this is entirely absent. Dog serum in one case dissolves the red cells of cats very strongly, in another case it does not do so at all. The action of rabbit serum on guinea-pig blood shows a special variability.

A very interesting example is afforded by lamprey serum, which, as is well known, possesses an extraordinarily toxic action for laboratory animals in general and also for red blood-cells *in vitro*. Dr. Schönlein of Naples, whose recent death we lament, was kind enough to experiment with this for us. His investigations showed that the serum of a not inconsiderable number of lampreys possesses no toxic action at all, so that it could be injected into rabbits intravenously in amounts of 2 cc. without any damage whatever.

It is clear that this extensive variability enormously increases the difficulties in investigating these sera. Thus on repeating the well-known experiment of Buchner, whereby a mixture, in certain proportions, of dog and rabbit sera loses its hæmolytic property for guinea-pigs in the course of twenty-four hours, we were able to completely confirm Buchner's results in three cases, while in five other cases the hæmolytic effect was only more or less lost.

We believe that all these investigations support the view we have already expressed regarding the nature of the complex poisons of the blood-sera. v. Dungern (Muench. med. Wochenschr., 1899, No. 14), basing his action on some new experiments of his, has accepted our views. We can content ourselves, therefore, with merely mentioning another view, recently expressed by Bordet<sup>1</sup>. He has confirmed the statements made by us regarding the fixation of the specific immune body by means of the corresponding erythrocyte, and he has ad-

<sup>1</sup> Annal. de l'Institut. Pasteur, April 1899.

mitted that the fixation process is connected with the solvent process, but he believes that the nature of this connection requires a special hypothesis:

"On pourrait rapprocher, si une comparaison un peu grossière était permise, la modification apportée par la substance sensibilisatrice [our immune body] sur le globule, de celle qui consisterait à changer la structure d'une serrure, de façon à y permettre l'introduction facile d'une ou de plusieurs clefs qui n'y entraient pas auparavant ou n'y pénétraient qu'avec difficulté. Deux clefs suffisamment semblables enteront dès lors indifféremment."

One could therefore picture the mode of action of the two substances as it is conceived by Bordet to be like a safety lock which requires two keys to open it, of which the first is necessary in order to make the main lock accessible.

Against this mechanical conception it can be urged that the keys do not fly into the lock of their own accord, but that certain *forces* are necessary to effect this. Our theory supplies a very simple explanation for this; the driving force is the chemical affinity between the fitting groups. The entire line of experiments made by us was designed to show whether the two substances, together, combined with the blood-cells at one place or whether, separately, at two different places. Our decision was determined by the demonstration that the addiment was in no way fixed by the red blood-cells. Had Bordet repeated not only one of our experiments, but the entire series, the inapplicability of his hypothesis would have become evident to him.

If active immune serum is treated with red blood-cells, at 0° C. as described in our first article, thus fixing the immune body, the lock, according to Bordet, is made accessible, i.e. the conditions are fulfilled whereby the addiment (Bordet's alexin) could penetrate to the blood-cells. As a matter of fact, however, under these circumstances the addiment does not do so. This, as well as the new facts mentioned in the present article, harmonize best with our theory.

If, however, this mode of action of the lysins is accepted, it will be impossible not to hold the same views regarding the living protoplasm, and assume in this the presence of side-chains of peculiar character which are designed to grasp highly complicated substances. It must further be assumed that these side-chains, beside their grasping group, are endowed with a second group which, by fixation of peculiar ferments, effects a digestive action.



### III. STUDIES ON HÆMOLYSIS.<sup>1</sup>

#### THIRD COMMUNICATION.<sup>2</sup>

By Professor Dr. P. EHRLICH and Dr. J. MORGENROTH.

By injecting one animal with the cells of another, we can produce substances in the serum of the first, which have a specific damaging or destructive influence on these cells. This possibility has within a short time extended the theoretical doctrines of immunity in various directions. First Belfanti and Carbone showed that the serum of animals, after these had been treated with blood-cells of a different species, acquires a high degree of toxicity for just this species. Shortly afterward, Bordet was able to demonstrate that this toxicity in corpore corresponds to a specific hæmolysis in vitro. This was confirmed independently by von Dungern and Landsteiner by experiments published somewhat later, and further by those of our own mentioned in previous communications. The result of the experiments is always, that, following the introduction of red blood-cells of one species into the organism of another, a hæmolysin is formed which so injures the blood-cells of the first species that their hæmoglobin goes into solution. Bordet also showed that this hæmolysis depends on the action of two substances in the hæmolytic serum.

The importance of this subject, due specially to the complete analogy between the hæmolytic and the bacteriolytic processes, led us to a detailed study of the mechanism of these processes. We were able to show that the substance produced by immunization, the immune body, possesses a maximum chemical affinity for the corresponding blood-cell. This affinity is due to the presence of a specific combining group in the molecule of the immune body, which fits to a corresponding group in the protoplasm of the erythrocyte. Beside this, the immune body possesses a second combining group

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<sup>1</sup> Reprint from the Berliner klin. Wochenschr. 1900, No. 21.

<sup>2</sup> See pages 1 and 11.

which fits to a group in a ferment-like body of normal serum, namely, the complement (addiment). By virtue of these two haptophore groups, the immune body functionates as a coupler or *interbody*, carrying the action of the complement over onto the red blood-cells,

In order to facilitate expression, *that combining group of the protoplasmic molecule to which the introduced group is anchored* will hereafter be termed *receptor*. The side-chain, for example, which combines with the tetanus toxin in the organism is such a receptor. The tetanus antitoxin itself is nothing but the surplus of receptors thrust off into the blood. Similarly, that complex which *later* functionates as immune body is a receptor *before* being thrust off.

In the further course of these investigations it has been found that the function to produce peculiar antibodies analogous to immune bodies is not confined to bacteria and erythrocytes. Cells of the most varied kind, provided they are absorbed, excite the production of immune bodies, in conformity with the requirements of the side-chain theory. Landsteiner, Metchnikoff, and Moxter succeeded in producing an immune serum against spermatozoa; von Dungern, a specific serum which acted on ciliated epithelium; and Metchnikoff, an immune serum against leucocytes and kidney epithelium. Here also in the cases examined for this purpose (v. Dungern, Moxter) it could be shown that the specific active substances are of complex nature, consisting of an immune body and a corresponding complement, and that the immune body possesses a specific affinity for the corresponding cells.

The great theoretical significance of these investigations which open up a new field to the study of immunity is clearly apparent, but whether in the near future they will have any practical results remains to be seen.

In the pursuit of these studies, we were led to extend our researches into another direction which seemed to us of special importance in the understanding of pathological processes.

The experimental investigations thus far made have dealt exclusively with the changes in the serum which occur when an animal is made to absorb *foreign* cell material. This mode of experiment, however, is not limited in any way by the nature of the subject, but is dependent entirely on the will of the experimenter, and it therefore lacks all physiological analogy.

In pathology, the changes foremost to be considered are those resulting from the absorption, by an organism, of *its own* cell mate-

rial. Such occasions are presented by many different diseases. Keeping to the blood, for example, if an individual suffers a considerable subcutaneous hemorrhage or one into a body-cavity, or if part of his blood-corpuscles are destroyed and dissolved by certain blood-poisons, the essential conditions, just as in an experiment, are given for the reactive formation of substances possessing specific injurious affinities for these blood-cells. The same, however, can apply to other tissues; for every acute atrophy of an organ's parenchyma can lead to the absorption of cell material and to its consequences. The conditions necessary for the development of specific cell poisons may be presented by various circumstances, thus, when, spontaneously or under the influence of arsenic, large lymph-gland tumors are absorbed; when a struma melts and disappears under specific treatment; when the white blood-cells, owing to the action of toxins or other substances, are caused to disintegrate; when, owing to certain metabolic or infectious diseases, acute atrophy of the liver ensues, etc. We shall further have to assume that these conditions can be fulfilled, in a wider sense, when, under the influence of certain general diseases, there occurs active dissolution of organized material of any kind instead of atrophy of a single organ.

It is therefore of the highest pathological importance to determine whether the absorption of its *own* body material can excite reactive changes in the organism, and what the nature of these changes is. The simplest conditions and those most accessible to experimental study are those which arise on the absorption of blood-cells. But here we face a curious dilemma. If an animal organism, when injected with blood-cells of foreign species, always produces a specific hæmolysin for each of these species, it must surely be following a natural law; and it is improbable that this law which applies in any particular number of cases should be suspended in the case of blood-cells of the same individual. On the other hand, it is not to be denied that the formation of such hæmolytic substances would appear dysteological in the highest degree. For example, if, in an individual who has had an extensive hæmorrhage into a body-cavity, the absorption of this blood caused the formation of a blood poison which destroyed the rest of the blood-cells, this would be a phenomenon whose actual occurrence lacks any clinical evidence whatever and one which no one is willing to accept.

It cannot be doubted that the organism seeks a way out of this difficulty by means of certain regulating contrivances, whose deter-

mination will be of the highest interest. To be sure the study of this question offers considerable difficulties, difficulties through which previous experiments in this direction have been brought to naught. (Belfanti and Carbone, Bordet.)

We have from the beginning maintained that it is possible to gain an insight into these processes, only when any changes occurring in the serum are determined by means of frequent and progressive examinations. Small laboratory animals, because of the amount of blood required for these continuous examinations, are therefore unavailable, and hence we selected goats as being best adapted for these experiments.

After it had been determined that a single injection of a large amount of blood sufficed to produce the specific hæmolytic substances in the serum, we usually injected our animals once with a large amount of goat-blood. (800-900 cc. for a goat of 35-40 kg.) In order to overwhelm the body as rapidly as possible with the constituents of the blood-cells, we made use of intraperitoneal injections. For the same reason we thought it best not to inject intact blood-corpuscles, but to inject blood which had been made laky by the addition of water. We argued that blood-cells of the same species as the animal injected would be destroyed very slowly in the peritoneal cavity of this animal, and that consequently the absorption would be so gradual as to prevent the occurrence of what may be termed an "ictus immunisatorius." From the second or third day on, we withdrew samples of serum from the animals so treated, and tested the solvent action on the blood of numerous other goats. Our method generally was first to determine whether any indications of hæmolytic action were present. For this purpose a drop of normal goat blood was allowed to fall into undiluted serum of the treated goats, and the occurrence of any red coloration looked for. If this test was positive, we proceeded to test the hæmolysin in the usual manner by adding decreasing amounts of this serum to tubes containing 1 cc. of a 5% mixture of goat-blood in 0.85% salt solution.

With these preliminary remarks we proceed to our first positive test (February 16, 1900). The subject of this was a strong male goat, buck A, weighing 33.5 kg., into whom there were injected intraperitoneally 920 cc. goat-blood (mixed from the blood of goats 1, 2, and 3) made laky by the addition of 750 cc. water. From the second day on, small amounts of blood were withdrawn daily for

the purpose of obtaining serum. This serum, as we had anticipated, never showed a trace of hæmoglobin coloration. As early as the second day, a slight solvent action for the blood of goats 4 and 5 was developed. A drop of the blood allowed to fall into the undiluted serum of buck A suffered partial solution, so that after the blood-corpuscles had sedimented, the serum remained slightly tinged with red. By the fifth day the solvent property had increased considerably; 0.5 cc. serum completely dissolving 1.0 cc. of the 5% blood-mixture of goat No. 4. By the seventh day the action had reached its maximum. 0.3 cc. serum produced complete solution (No. 4); 0.07 a just appreciable effect.

As we now had at our disposal a sufficient amount of hæmolysin, we sought to determine whether this hæmolysin dissolved all goat blood-corpuscles without exception. We found that of nine goats which we examined, the majority were markedly sensitive to this hæmolysin. Thus goats Nos. 1, 2, 4, 5, 6, and 9 were highly sensitive; two goats, Nos. 3 and 8, somewhat less so; and only one, No. 7, (which had previously been treated for some time with the expressed juice of eel muscle,) showed so slight a susceptibility that even undiluted serum failed to cause strong solution.

After noting these results it was important to determine the behavior of the blood-cells of this buck toward the hæmolysin of his own serum. If a drop of blood was added to the serum, in vitro, not even a trace of solution occurred. These blood-cells then were entirely insusceptible to the hæmolysin of their own serum, as had already been indicated by the absence of hæmoglobin coloration in the freshly drawn serum.

If we designate the specific hæmolysin developed by the injection of blood of foreign species as *heterolysin*, then we must designate the hæmolysin due to the injection of blood of the same species as *isolysin*. In no case, however, and this is to be emphasized, are we here dealing with an *autolysin*, i.e. a lysin which dissolves the blood-cells of the animal in whose serum it circulates. However, such a condition is not at all a matter of course, and the question arises why the isolysin in this case does not also functionate as autolysin.

The toxins as well as the hæmolysins can act only when they are anchored by certain haptophore groups, the receptors, whereby the action of the poisons is concentrated on the cells possessing these receptors. If these groups are lacking, the poison has no point of

attack. We have already demonstrated that a hæmolysin, or rather its immune body, is anchored by the erythrocytes, and the solution of the above question therefore becomes very easy. To begin, we have determined that the isolysin behaves like a typical hæmolysin of the well-known kind. It loses its action by being heated for half an hour to 55° C. (destruction of the complement) and is reactivated by the addition of a corresponding amount of normal goat serum.

Next we have determined that the immune body of the isolysin is bound by the *susceptible* blood-cells in typical fashion; that the blood-cells of the immunized animal, however, take up only traces of the immune body in vitro, amounts far less than those taken up by the almost insensitive blood-cells of goat No. 7. This phenomenon can at once be ascribed to a slight mechanical absorption. We see, therefore, that the serum's own insensitive blood-cells are incapable of anchoring the specific immune body of the isolysin.

This result can be explained in either of two ways. It may be assumed that the blood-cells lack this receptor entirely, or that, although the cells possess the receptor, the affinity of this had already been satisfied by the immune body in the circulation. In the latter case, however, it is incomprehensible why the blood-cells were not dissolved by the complement also circulating in the blood. Further reasons against the latter assumption will be apparent later, and so we shall at once discuss a series of facts which, according to our views, demonstrate that the insusceptibility of the blood-cells in this case is due to an *absolute lack of these receptors*.

Assuming that a given toxin, in an organism, finds receptors which anchor it, the injection of this toxin will be followed by the production of a corresponding antibody. If, however, an organism lack receptors for this poison, the first essential for the production of an antibody will be wanting. In the development or non-development of antibodies we shall have an indication of the presence or absence of receptors.

Now the hæmolysins belong to the class of poisons which produce antibodies. We ourselves have demonstrated that the normal hæmolysins of dog's and goat's serum, when injected into a foreign animal body, excite the production of antihæmolysins. The question was whether the isolysin when injected into the organism of *other* goats would be able to cause the production of an *anti-isolysin*. In order to save material we injected a young goat (No. 10), whose

blood-cells we had previously shown to be very sensitive to the isolysin, several times with considerable quantities of serum A. As a matter of fact an antibody was developed, so that 0.4 cc. of the serum thus obtained were able to protect 1 cc. of a 5% sensitive goat-blood-cell mixture against solution by isolysin A (0.5 cc.). The blood-cells of this same goat No. 10, on the contrary, after they had been repeatedly washed with physiological salt solution to free them from serum, proved just as susceptible to the isolysin as before. Hence it follows that the isolysin here concerned, isolysin A, causes the production of antilynsins in the body of the same species when it finds fitting receptors.

From this we conclude that the insensitiveness of the red blood-cells can only be due to the lack of receptors for the isolysin. A further conclusion must be that these receptors are not present in any other tissue of buck A, *that they are absent in the entire organism*, for otherwise there should have been a formation of anti-isolysin.

It goes without saying that we repeated these experiments on a large number of animals in order to exclude all accidental phenomena. In the course of these experiments we noted numerous and interesting variations in the reaction to isolysins.

Of special interest is goat B, which had been treated exactly like buck A. At first it seemed as though the experiment with this animal would run an entirely different course, for during the first fourteen days we were unable to detect even a suggestion of an isolysin. The red cells, however, remained completely sensitive to the isolysin derived from buck A. Then suddenly on the fifteenth day after the blood injection a hæmolysin made its appearance, one which acted on goat blood quite as strongly as the isolysin of buck A. The animal's own blood-cells were just as insensitive to this hæmolysin as were those in the first experiment to theirs. Here also, then, we were dealing with an isolysin, not an autolysin. The sensitiveness of the blood toward isolysin A continued. We now examined the majority of our goats in order to determine their sensitiveness to this isolysin, and found that some animals which were highly sensitive to isolysin A were very slightly sensitive to isolysin B, and vice versa. The blood of buck A occupied a peculiar place. It was as completely insensitive to isolysin B as it was to that of its own serum.

From the behavior of the blood of the various animals toward these two isolysins, it was clear that these isolysins were essentially

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different. This was positively proven by the fact that the anti-isolysin A was entirely ineffectual against isolysin B. The difference between these two isolysins is further illustrated by the difference of the intervals between blood injection and isolysin formation. In the one case this was only a few days and in the other fourteen days. That the injection of the goat blood should result in the formation of two entirely distinct and easily differentiated isolysins was certainly a remarkable phenomenon. And yet this did not exhaust the multiplicity of the isolysins.

In a third goat, C, (injected on the same day as B and with similar amounts of the same blood,) a hæmolysin C appeared on the seventh day which again differed from isolysins A and B. This, furthermore, proved itself an *isolysin*, for the blood-cells of the animal were entirely insensitive to its action, though they were sensitive to isolysins A and B. This fact shows that isolysin C differed from isolysins A and B. It is specially noteworthy that, although the two goats B and C were injected at the same time with similar amounts of the same blood, they should develop *different* isolysins. This observation is particularly important because it shows that the constitution of the isolysin is dependent on the individuality of the animal in which it is developed.

It is also very remarkable that these three isolysins, A, B, and C, were able to destroy not only goat blood-cells, but also those of sheep. The sheep erythrocytes therefore possess three different groups which are identical with those of these goat blood-cells, or at least are closely related to them. On the other hand still another isolysin, D, does not dissolve sheep blood-cells.

After having observed three different isolysins in three different goats, we are in no wise to assume that this exhausts the possibilities.<sup>1</sup> On the contrary, it seems highly probable that by further experiments we shall come to know other isolysins. Nevertheless it must not be assumed that this variation of the isolysins is unlimited. It is to be expected that a sufficient repetition of the experiments will finally lead us to recognize a certain cycle of constantly repeating types. The attainment of this goal, however, is rendered very

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<sup>1</sup> *Note on revision.*—In the mean time we have obtained a fourth isolysin, D, which differs from isolysins B and C in the fact that it dissolves the blood-cells of B and C. Erythrocytes of A are not dissolved, but the isolysin differs from A in its behavior to various normal kinds of goat blood. The behavior of isolysin D toward sheep blood has already been mentioned.



tedious by the fact that in some cases in which the production of an isolyisin is attempted after the method already outlined, no isolyisin is formed. We have records of a number of goats in which the injection of goat blood produced apparently no effect whatever; among these is one which was injected with its own blood.

The differences in the isolyisins in their dependence on the injected blood and on the individuality of the treated animal, the fact that there is formed always an *isolyisin*, not an *antolyisin*, the special conditions governing the formation of the anti-isolyisins, the failure of the isolyisin reaction in certain cases, all these make the problems connected with the above facts appear very complicated, and make it necessary now to analyze these more closely.

Every red blood cell possesses a large number of side-chains with haptophore groups, each of which is able to combine in the animal body with fitting receptors. Let us, in our own case, designate such a group of the injected goat erythrocytes as group *a*, and a corresponding receptor as receptor *a*. There will then be presented two possibilities. First is the possibility that the *a* receptor is entirely absent in the organism of the goat into which the blood is injected. If this be the case, there is lacking the essential condition for the formation of any reactive product, and the result of the injection will be entirely negative.

If, however, the second possibility obtains, and *a* receptors are present in the body of the animal injected, there are again two ways in which the reaction may proceed: (1) the *a* receptors *exclusively* may be present; (2) besides these, the organism may contain the same group *a* which is present in the injected blood cells.

We shall study these two cases separately and begin with the simpler, in which only *a* receptors are present. In this case the conditions for the formation of a hemolyisin are given and the banding, hyper-regeneration, and final throwing off of the *a* receptors will follow. This newly formed immune body, in conjunction with the complement always normally present, will dissolve all those goat blood-cells, and only those, which possess the group *a*. But as this group *a*, according to our assumption, is completely absent in the organism of the animal itself, the immune body fails here to find any point of attack. The immune body therefore will accumulate in the blood without hindrance and without causing the slightest damage to the organism. This case is the one which applies to the examples of isolyisin formation described by us, for it is the

only one which fulfills the conditions necessary for a permanent existence of a free hæmolysin.

The course of the reaction, however, is entirely different in the second case, i.e., when the group  $\alpha$  of the foreign blood-cells which fits into the receptor group is found also in the organism of the animal injected, being present in its blood-cells and tissues. In this case, groups fitting to one another would be present in the same organism. A pregnant example is seen in this, that both the rennin and the antirennin group may occur simultaneously in the organism. In fact we believe that this simultaneous occurrence of such corresponding groups is a very frequent phenomenon in the economy of the organism, and that it occurs especially in those cases in which a certain cell is dependent for its nutrition on the products of a different kind of cell.<sup>1</sup>

If this is the case, i.e., when group  $\alpha$  is present in the organism beside the receptor group, the first phase will proceed just as in the first case. There will be a binding, regeneration, and thrusting-off of the receptor as immune body. The difference in the course of the reactions becomes manifest in a second phase in which these thrust-off receptors are taken up by group  $\alpha$ .

Under certain circumstances this might lead to serious injury, namely, when the thrusting-off of the receptors as immune bodies occurs so suddenly that the organism is overwhelmed, the red blood-cells anchoring the receptor group and being dissolved by the ever-present complement. In this case, then, an *autolysin* could develop. But this result need not of necessity ensue. It can be prevented, for example, if at first only small amounts of the liberated receptor

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<sup>1</sup> In contrast to this we shall have to assume that *singular* haptophore groups occur wherever it is designed to catch hold of certain exogenous constituents of the nourishment. In immunization it is of some consequence whether a singular group functionates as receptor, or one which corresponds to another. The former is probably the case with the toxin, and this permits of an extraordinary increase in the production of antitoxin, being limited by no regulating contrivance. If, however, the antigroup is present in the organism, owing to secondary influences, a regulatory production of new antigroups will occur. This might be the reason why it is apparently impossible to increase the production of antirennin to any desired degree. The antirennin finds the corresponding rennin group in the organism and causes the production and thrusting off of this group. As a result of this series of changes we find at one time that the serum of an animal contains free antirennin, at another time that rennin is being excreted by the urine.

(immune body) reach the tissues. This would effect a production and thrusting-off of the corresponding group  $\alpha$ , which would then circulate as an antiautolysin and serve to switch the autolysin thereafter formed, away from the blood-cells. Be this as it may, whether the organism be injured as a result of an acute flooding with the liberated receptors, or whether this injury be prevented by the slow course of the reaction, the end result in the second case will regularly be a development of an *antiautolysin*.<sup>1</sup>

The three possibilities, therefore, which present themselves on the injection of blood of the same species are: 1, *the failure of any formation of hæmolysin*; 2, *the formation of an isolysin*; 3, *the development of an antiautolysin*.

Each haptophore group of the red blood-cells (and we have reason to assume a large number of different groups in each erythrocyte of every species) will have to react, in the animal body, according to the above scheme. This leads to a large number of possibilities. If, for example, an injected blood-cell possesses three haptophore groups,  $\alpha$ ,  $\beta$ ,  $\gamma$ , it will be possible for  $\alpha$  to cause the development of an isolysin,  $\beta$  an antiautolysin, while  $\gamma$  produces no effect whatever.

This, of course, complicates the problem extraordinarily. A multiplicity of variations is presented whose complete investigation would require a great deal of time and labor. The three cases above-mentioned, however, amply suffice to explain all our observations thus far. The differences in the three isolysins previously described are to be ascribed to the action of three different haptophore groups of the blood-cells; and the fact that the same blood injected into two animals causes the development of different isolysins is to be explained by the individual differences in the receptors. Finally, the failure of any isolysin reaction whatever would correspond to an absence of suitable receptors.

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<sup>1</sup> The cases here discussed are of general significance for the question whether hæmolysins exist at all, and they determine also the conditions under which the hæmolysins of normal serum are capable of existence (see also the second communication, pages 11-23). The fact that a normal hæmolysin dissolves the blood-cells of foreign species but spares its own blood-cells, that, for example, dog serum dissolves guinea-pig blood, rat blood, goat blood, sheep blood, etc., but not dog blood, is only a single instance of the above-mentioned general law that autolysins are not capable of existence in an organism; for the presence of receptors, which is essential to the production of autolysins, would, if the autolysins should develop, soon result in a compensation by means of anti-autolysin formation.

Though the existence of the antiautolysin is theoretically possible, we have thus far been unable to demonstrate it. To do it would first be necessary to get hold of an appropriate autolysin. The possibility of getting this, however, is only conceivable in favorable cases where the autolysin might be produced critically and in large amounts. This certainly did not occur in the cases observed by us, and we were therefore compelled to try a different method to demonstrate such an antibody. We know of a number of hæmolysins which dissolve goat-blood and which therefore act on certain haptophore groups of the goat blood cells. It is conceivable that one of these haptophore groups is identical with that of the autolysin sought for, and that an antiautolysin fits this group.<sup>1</sup>

With this end in view we have made a number of experiments and tested the action of our inactive goat serum on the goat-blood dissolving action of dog serum, pig serum, and goose serum and also the serum of a rabbit treated with goat blood. The results, however, were not positive. From this, of course, we are not to conclude that antiautolysins are not at all present in these cases. We shall rather extend and vary our experiments in all possible directions until a lucky coincidence leads us to find a fitting hæmolysin.

Perhaps the most important of the questions thus presented is whether this deficiency of binding groups in the red cells is performed or whether it is due to a new regulating power of the organism. In the latter case this power would be suited in the highest degree to protect the body even without the formation of an antiautolysin.

In one case, to be sure (goat E), it seemed as though the insensitiveness was developed only in response to the blood injection. The blood-cells of this goat (the goat had been repeatedly injected) were primarily sensitive to isolysins A and B. After the injection there developed a complete insensitiveness to isolysin B, although the sensitiveness to A remained. In this case an isolysin was developed, so that if accidental circumstances are excluded, it appears as if under the influence of this blood injection a direct change in the destruction of the binding groups had taken place.

We may perhaps also assume that the complete insensitiveness

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<sup>1</sup> The multiplicity of the combining groups of the blood-cells is well illustrated by the blood of buck A. This blood is insensitive to the isolysins mentioned. Independently of this, however, it retains complete sensitiveness to hæmolysins of a different origin, pig serum, goose serum, specific goose serum from rabbit.

of buck A to isolysin B is a secondary one, due to the treatment; for thus far, among the many normal goats examined, we have failed to find a single one whose blood-cells are completely insensitive to isolysins A or B.

These phenomena require further and more extended investigation, and in this we are at present engaged.

In closing we should like to point out that the difference between isolysins and autolysins emphasized by us makes several recent attempts directed to the solution of certain pathological processes, particularly those of autointoxication in man, appear questionable. It has frequently been ascertained that serum secretions and excretions of the diseased body are poisonous in animal experiments, and the conclusion has been drawn that the substances to which this poisonous action is due must exert an injurious effect on the organism of the patient. From the above analysis we see that this conclusion is not at all imperative. If, for example, the serum of a scarlet fever patient is especially toxic to guinea-pigs, it is possible that the same may be absolutely harmless to the patient himself. Even if one demonstrates that the serum of anæmic individuals dissolves the blood-cells of other individuals, it does not prove that this property is of any significance for the origin of the anæmia. On the contrary it is highly probable that this hæmolysin is only an isolysin and not an autolysin.

The above experiments may suffice to show how very complicated the conditions are when the material of its own body is absorbed by an organism. Drawing a general conclusion, however, we may say that such an absorption, which as already stated extends to the greatest variety of cells and occurs in numerous instances, will not as a rule lead to permanent injury of the organism, owing to the formation of reaction products. Only when the internal regulating contrivances are no longer intact can great dangers arise. In the explanation of many disease phenomena it will in the future be necessary to consider the possible failure of the internal regulations as well as the action of directly injurious exogenous or endogenous substances.

#### IV. CONTRIBUTIONS TO THE STUDY OF IMMUNITY.<sup>1</sup>

By Dr. von DUNGERN, University of Freiburg, Germany.

##### A. New Experiments on the Side-chain Theory.

THE combining experiments of Ehrlich and Morgenroth<sup>2</sup> showed conclusively that the two components of an immune serum necessary for hæmolysis and first demonstrated by Bordet, namely the immune body which withstands heating to 56° C. and the complement (addiment) which is present even in normal serum, can under certain circumstances exist in a serum side by side, uncombined. The immune body possessed a strong affinity to the blood-cells to which it specifically belonged, being anchored by these cells at 0° C. and thus separated from the complement, which latter remained in the serum. The complement was abstracted from the serum by the erythrocytes only at higher temperatures provided the immune body was present at the same time. When the latter was absent the blood-cells failed to combine with any complement whatever. The complement, therefore, because of its lack of affinity, was unable to act on the blood-cells, and likewise the mere anchoring of the immune body by the blood-cells, without the presence of the complement, was unable to effect any hæmolysis. The most plausible explanation for these facts was this, that solution is effected by the complement, but that this substance first requires the immune body to enable it to lay hold of the blood-cells.

Bordet<sup>3</sup> has assumed that the immune body, independently of the complement, combines with the substance of the erythrocyte and so changes this that it (the erythrocyte) now combines with the complement. Against this assumption must be urged that as a matter of fact there is a definite relation between immune body

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<sup>1</sup> Reprinted from the *Münchener med. Wochenschrift*, No. 20, 1900.

<sup>2</sup> See pages 1-23 of this volume.

<sup>3</sup> *Annales de l'Institut Pasteur*, 1899, No. 14.

and complement of the same species. An immune serum inactivated by heating to  $56^{\circ}\text{C}$ . can always be reactivated by the addition of fresh blood serum from an animal belonging to the same species as that from which the immune serum was derived. The complements of other species of animal, however, reactivate this immune body in the most divergent manner.

The results of the combining experiments were readily harmonized with the requirements of the side-chain theory. The immune body is nothing but a side-chain with two haptophore groups, which has been produced in excess and thrust off into the blood. One of these haptophore groups possesses a strong chemical affinity for the corresponding group of the erythrocyte, and ordinarily it serves to anchor nutritive material possessing corresponding haptophore groups to the cells. The other haptophore group is able to combine more or less completely with complement present in the serum. It is probably designed to collect from the blood plasma the ferment-like complement, which, by splitting up the nutritive substances, makes their assimilation possible.

There is, however, another view to take of these phenomena. It is comprehensible that the cell, as such, produces the two components necessary for hæmolysis simultaneously and in relation with each other, in such fashion that in the assimilation of the substances anchored, it constantly produces the complement required by means of its own activity and does not depend on the supply from without, from the blood plasma. The assumption of such a complex system—in which two members so intimately connected are yet so readily dissociated—offers difficulties which it is unnecessary to discuss further, especially because, as will be seen later, experiments have precluded this possibility.

If, however, the side-chain theory is correct we shall expect:

1. That immune body and complement are not present in the immune serum in equivalent proportions, but that quantitatively they may be independent of each other.

2. That the same group of the red blood-cells which in hæmolysis combines with the immune body causes the production of the immune body.

3. That cells which possess such form of complex side-chains are enabled by the presence of the complementophile groups to abstract complement from the blood serum.

1. The question whether in the immunity reaction only the inac-

tive immune body is produced, which then combines secondarily with the complement present in the blood, or whether the two substances reach the circulation together, can under favorable conditions be answered by an exact quantitative analysis of the immune serum for immune body and complement.

I have therefore treated a number of rabbits with cattle blood, cow's milk, and tracheal epithelium of cattle, and examined the hæmolytic immune sera thus obtained for their exact content in immune body and complement. Corresponding to the material injected, the erythrocytes of cattle were always used as a reagent. The method employed was the same in all cases; decreasing amounts of the various blood sera were mixed, each with one-half cc. 5% cattle blood dilution (in 0.8% NaCl solution), the mixture was kept at 37° C. for two hours and tested for hæmolysis. It was then very readily proven that an equivalence between immune body and complement does not at all exist.

If such an equivalence were present, the immune body of the fresh immune serum would be completely saturated with complement and would not become more active by the further addition of complement. The experiments demonstrated the contrary, for in some cases the power of the immune sera was markedly increased by the addition of normal rabbit serum, which, in the doses employed, was not itself able to effect the slightest solution of the cattle blood-cells. For example, if the fresh serum of a rabbit which had been treated with cattle blood was able to make ten times its volume of a 5% cattle blood mixture completely laky, the same serum on the addition of a sufficient amount of complement was able to dissolve 320 times its volume. On comparing the various immune sera with each other, it is seen that this increase in the hæmolytic action on the addition of complement is in direct proportion to the amount of immune body present.

*The experiments therefore prove that quantitatively the immune body is entirely independent of the complement.*

We can, however, go further and determine quantitatively the exact amount of complement contained in the normal serum on the one hand and in the immune serum on the other.

The amount of complement contained in the various normal sera was determined by always testing with the same amount of a blood immune body. *In fixing such a standard serum it is only necessary to take as a measure the action of an immune body saturated with*



*complement*, for equal amounts of *immune body* act differently with different amounts of complement. In all my tests on the amount of complement contained in a serum, I used so much inactivated blood immune serum that the immune body, when saturated with complement, could dissolve sixteen times the amount of blood present.

The experiments demonstrated that the amount of complement contained in normal rabbit serum is fairly constant, and even in different animals is not subject to great fluctuations. Proceeding as just described, it was found that complete solution took place in all cases on the addition of  $\frac{1}{40}$  to  $\frac{1}{20}$  cc. normal serum. Within definite limits therefore the complement in rabbit blood seems fixed.

The amount of complement contained in *immune serum* could be determined by comparing the hæmolytic action of the fresh serum with its action, after inactivation (by heating for twenty minutes to  $56^{\circ}$  C.), on the addition of various amounts of normal rabbit serum, the complement content of which was known.

The serum of the rabbits treated with cattle blood, serum which had been shown to contain such a large excess of immune body, was tested 1, 2, 3, 4, 11, and 14 days after the injection and failed in all of the numerous cases to show even a trace of increase in the amount of complement it contained. A peculiar state of affairs is thus presented. Since hæmolytic action is dependent on the immune body so far as this can combine with the complement, we see that the hæmolytic action of fresh immune serum can be increased only up to a certain point, determined by the amount of complement contained in the normal blood serum. All additional amounts of immune body formed in the course of the immunity reaction therefore remain latent, and manifest their action only when the immune body is brought into combination with greater amounts of complement. This can be done artificially, in test tube experiments, by the addition of normal serum, or experimentally by injecting the immune body into a suitable animal body.<sup>1</sup>

*Immune serum therefore differs from normal serum only in its content of inactive immune body.* Accordingly, in the immunity reaction, only inactive immune body is produced by the cells in excess. This

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<sup>1</sup> So also the earlier observations, as those of R. Pfeiffer, on cholera serum, my own on epithelial immune serum, and those of Moxter on antispermatozoa serum, in which the immune sera, in themselves little or not at all active, showed their full power when injected into fitting animal bodies, are to be explained by the relative poverty of these sera in preformed complement.

result is easily understood on the basis of the side-chain theory, if we assume that the production of the complement is entirely independent of the binding of the injected substances by the side-chains, and is probably referable to other cells. If the production and thrusting off of the particular side-chains exceeds a certain limit, these side-chains will fail to find in the blood serum any more complement whose haptophore group is still available. The disproportion between immune body and complement then sets in. This will be most marked in those cases in which the normal serum contains but little complement and in which a considerable production of immune body can be effected.

2. Certain experiments which I have described in a previous communication regarding globulicidal action of the animal organism<sup>1</sup> led me to the view that the immune body combines with a particular group of the blood-cells and thus leads to their solution. This conception was based on the fact that a specific affinity exists between erythrocyte and the corresponding immune body, which affinity must be the same in the production as in the action of the immune body. According to the side-chain theory just this affinity is the driving force which on the one hand anchors the corresponding group of the erythrocyte to the preformed side-chains (such side-chains when thrust off constituting the immune body), and on the other, in hæmolysis, anchors the immune body, and with it the complement, to the blood-cells.

It must always be conceded to the opponents of this view that the evidence to prove such complicated processes as will develop in the cells after inoculations of blood into an animal body will not, perhaps, be absolutely conclusive. If one were willing to forego an explanation of the specificity, one could assume that the immunity reaction is based on an increase of the normal function of certain cells whose products are formed without requiring a certain group to fit into a corresponding one.

It was therefore of great interest to be able to show experimentally that the group which in hæmolysis combines with the immune body actually gives rise to the production of the immune body. This demonstration was effected by injecting blood together with inactivated blood immune serum.

If the development of the antibody is independent of the group

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<sup>1</sup> Münch. med. Wochenschrift, 1899, Nos. 13 and 14.

to which the immune body is attached, the immunity reaction will be exactly the same whether the injected blood is loaded with immune body or not. If, however, the production of the immune body is dependent entirely on the molecular group for which the immune body possesses a specific affinity, no immune body will be developed when a sufficient amount of inactivated blood immune serum is added to the injected blood, since the group is already occupied by immune body and no longer offers the cells a point of attachment.

The experiments completely confirm the latter assumption. *When the blood loaded with immune body was injected, no immune body whatever was developed in the injected animal;* whereas in a control rabbit, injected with exactly the same amount of cattle blood (30 cc.), but without immune body, so much was produced that the serum eleven days after the injection was able to dissolve completely eight times its volume of full blood provided sufficient complement was added.

This fact, like many others, speaks against the idea that the immune bodies or the analogous antitoxins are not reaction products of the organism but are derived by modification from the substances introduced, a view still maintained by certain high authorities. The phenomenon, however, is readily explained on the basis of the side-chain theory. Since the particular groups of the erythrocytes, which otherwise give rise to the immunity reaction, are already occupied by immune body, it is impossible for them to be bound by the side-chains, which are absolutely similar to the immune body.

3. According to the researches of Ehrlich and Morgenroth, the erythrocytes of sheep possess no affinity whatever for the complement of normal goat serum. If instead of sheep blood-cells, one employs those of cattle and allows them to act on rabbit blood serum, exactly the same thing will be observed; the rabbit blood serum, centrifuged after prolonged contact with the blood-cells, shows no diminution in the content of complement. *If, however, other cells, e.g., ciliated epithelium from the trachea of cattle, be mixed with rabbit serum, the result is directly opposite, the complement decreasing, and even under some circumstances disappearing entirely.* In like manner the rabbit serum may lose its complement through the action of other cells. In the case of various mammals and birds, every one of the organs tested—liver, spleen, kidney, testis, lung, and brain—was able to abstract more or less complement from the rabbit serum. Yeast cells and fission-fungi were also able to effect this. Especially remark-

able, however, is the fact that the body cells of the same animal are able to produce this phenomenon.

Exact quantitative examinations showed that there were distinct differences. The spleen and kidney of a rat, for example, were more strongly active than the same organs of a guinea-pig, while the liver tissue of the two species possessed equal activity; the spleen and kidney of the rat abstracted more complement from rabbit serum than did the same quantity of liver tissue, whereas in the guinea-pig the liver acted more strongly than the spleen, and the latter, again, more strongly than the kidney. Virulent cholera vibrios acted only one-quarter as strongly as the completely avirulent "cholera Calcutta." (The number of active individuals could not, of course, be regarded.) Yeast cells were weakly active, anthrax bacilli strongly so. In the case of anthrax bacilli I tested the action of heat on this property to abstract complement from rabbit serum, and found that it is not destroyed by heating the bacilli for twenty minutes to 56° C., but that it is destroyed by heating them for only a short time to 98° C. But the property of the cells to abstract complement from rabbit serum is lost not only through the action of heat, but also *when the particular cells previous to their mixture with rabbit serum have been allowed to remain in contact with another serum.* For example, 1 gm. finely crushed kidney tissue of cattle is mixed with 2 cc. cattle serum, allowed to act at 37° C. for half an hour and then separated from the serum by centrifuge. If 2 cc. rabbit serum are now added to the sediment, and this is allowed to stand for half an hour at 37°, it will be found on testing with cattle blood immune body that there is no diminution of complement content; but such a diminution does occur when, with exactly the same procedure, 8 p. m. NaCl solution is used in place of the cattle serum.

These phenomena are best explained by assuming that the cells in question, in contrast to the erythrocytes, possess groups which have a very close chemical relation to those of the complement which reactivates the cattle blood immune body. The affinity of the cells may, in fact, be greater for the complement than for any immune body directed against other cells of the same animal species. For example, if we add ciliated epithelial cells from the trachea of cattle to an immune serum derived from a rabbit by treatment with cattle blood, we shall under favorable circumstances find that the immune body has been partially, but the complement completely, abstracted

from the serum. In this, therefore, the combining relations are just the opposite of those found by Ehrlich and Morgenroth to exist between blood-cells and their corresponding immune body. The tracheal epithelial cells must therefore possess complementophile groups. The immune bodies, which according to the side-chain theory are only the side-chains thrust off into the circulation, are similarly supplied with complementophile groups. These facts speak for the correctness of the views of Ehrlich and Morgenroth, especially when we consider that a cell, corresponding to its many-sided functions, possesses not merely one kind of side-chain, but side-chains of the most highly developed form.

Mammalian erythrocytes in contrast to the tissue cells seem not to possess complex side-chains; and this is readily understood when we consider that the red blood-cells of these animals, being without a nucleus and unable to maintain their nutrition independently are not complete analogues of the tissue cells; and further that their conditions of nutrition, corresponding to their simpler functions, must be less complicated than those of the typical tissue cell. Among the living constituents of the body, the red blood-cells constitute the simplest case and are therefore particularly adapted to the solution of many special problems in immunity, as can be seen from the course of the last experiments.

The phenomenon, that body cells are able to abstract complement from the serum, furnishes us with a good explanation of the fact that immune sera are often so little active in an organism of a different species. The immune body, which in stronger concentrations is not saturated with complement, even when the immune serum is perfectly fresh, can lose its complement entirely in the body of an animal of different species; it will therefore become active only when it finds a fitting complement in the new organism. Hence in serum therapy it is advisable, as Ehrlich has proposed, to employ for purposes of immunization, animals closely related to man, and furthermore to search for *anthropostable* complements.

#### B. Phagocytosis and Globulicidal Immunity.

In a previous communication <sup>1</sup> I expressed the view that the specific increase of the globulicidal function of the organism, following the introduction of chicken and pigeon blood, is due to the action of the

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<sup>1</sup> Münch. med. Wochenschrift, 1899, Nos. 13 and 14.

serum and not to the activity of the phagocytes. That the taking up of the blood-cells by the phagocytes in the specifically treated guinea-pig is necessary for the solution of the blood-cells was excluded by the fact that hæmolysis is also effected in the peritoneal cavity of the animals apart from the phagocytic cells. Furthermore, a transference by the phagocytes of the substances necessary for solution was not suggested because the exudate, rich in leucocytes, which was produced in specifically immunized guinea-pigs by injections of an aleuronat mixture, showed a much smaller content of both immune body and complement than the blood which was poor in leucocytes.

Metchnikoff has objected to these experiments.<sup>1</sup> He states that aleuronat exudates contain principally microphages, whereas the blood is richer in macrophages, and that the latter alone are concerned in hæmolysis. I have therefore tested the spleen (rich in macrophages) of normal rabbits and guinea-pigs with a cattle blood immune body derived from rabbits in order to determine the amount of complement present. The experiments have demonstrated that the spleen also contains much less complement than the blood serum. For example, 1 grm. finely crushed spleen of an exsanguinated rabbit was mixed with 4 cc. of an 8 p. m. NaCl solution. This fluid, like similar mixtures derived from liver and kidney, when tested in the usual manner proved from eight to sixteen times weaker than the blood serum. Moreover, if the suspended organic particles were first washed with physiological salt solution, they yielded no complement whatever to the immune body. The spleen of a guinea-pig contained still less complement, although the serum of this same animal completely activated the cattle blood immune body derived from rabbits, and did so in even smaller quantity than the rabbit serum.

We must therefore in conformity with the side-chain theory look to the blood serum as the chief source of complement.

It is self-evident that the complement cannot originate in the blood plasma; it must, of course, be derived from some kind of cells. However, that it is especially abundant in the phagocytes is not at all borne out by the above experiments.

As for the immune body, Metchnikoff too believes this to circulate free in the blood plasma. According to his conception the macro-

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<sup>1</sup> Annales de l'Inst. Pasteur, 1899, No. 10.

phages yield this to the blood at the end of their intracellular digestion. Metchnikoff bases this view chiefly on his observations that the destruction of avian blood-cells in the peritoneal cavity of normal guinea-pigs is effected exclusively by the macrophages.

This statement is in direct opposition to mine, according to which even in untreated animals, the solution takes place free in the peritoneal exudate independently of the phagocytes. I believe, however, that these apparently contrary results can well be harmonized.

According to Metchnikoff the solution of goose blood-cells in the subcutaneous connective tissue of even non-immunized animals, is effected almost exclusively extracellularly. Hemolysis in this case must be due to a passage of complement and interbody from the blood into the subcutaneous tissues; this will naturally proceed more rapidly when, as a result of substances exciting inflammation, a stronger exudation ensues.

It would be very curious if the same conditions for the passage of hemolytic substances from the blood were not present in the peritoneal cavity. We know, for example, that Pfeiffer's phenomenon is especially marked in the peritoneal cavity. As a matter of fact, shortly after an injection of avian blood-cells into the peritoneal cavity of normal guinea-pigs, one always observes free nuclei, even when the serum has been removed from the cells by centrifugation. Of this I convinced myself by repeated observations. If one employs blood-cells of low resistance (chicken blood), and these in small doses, they will be degenerated and for the most part dissolved before they are taken up by the macrophages in any considerable number. When blood-cells of greater resistance are employed, and these in larger doses, the solution effected by the body juices will be comparatively slight and occupy more time. The taking up of these cells by the macrophages, which Metchnikoff in his splendid experiments was able to follow into the organs, will then come more to the front.

If therefore, as a result of experiments in which I used sensitive blood-cells in small doses, I underrated the significance of phagocytosis, Metchnikoff, through the conditions in his experiments, fell into the opposite error. The truth lies between these views; in the peritoneal cavity, according to the prevailing conditions, hemolysis can be effected free in the peritoneal exudate or in the interior of the macrophage.

In any case, phagocytosis is not essential for the development of



the immune body. The immunity reaction occurs even under conditions in which phagocytosis does not at all enter; and if, according to the observations of Metchnikoff, somewhat less immune body is produced after subcutaneous injections than after equal injections peritoneally, this may be explained as follows: In consequence of the slower absorption from the subcutaneous tissues, fewer cells come into contact with the group of the erythrocytes which excites the immunity reaction before an excess of immune body is thrust off by these cells into the blood. This immune body, of course, prevents any further combination of the group in question with other cells.

To what extent the phagocytes are concerned in the production of immune bodies must be determined separately in each case. No definite conclusions can be drawn from the experiments of Metchnikoff on guinea-pigs with goose blood-cells, for at no time did the organs of the specifically treated guinea-pigs show a stronger globulicidal action than those of normal animals, although such an increase in hæmolytic power was exhibited by the blood serum. But the observation has been made that even in normal animals the organs rich in macrophages are able, in contrast to other tissues, to dissolve goose blood-cells, and this observation is well adapted in this case to support the assumption of a special significance of the phagocytes for this function. However, that organs rich in macrophages effect hæmolytic action is not necessarily the case. For example, the spleen of a guinea-pig (1 grm. finely crushed spleen suspended in 1 c.c. of an 8 p. m. NaCl solution), in contrast to the blood serum of the same animal is not globulicidal for cattle blood.

Considering the large number of immune bodies, it will surely often occur that the phagocytes are preeminently concerned in the production of the immune body, especially since these cells frequently come into intimate relations with the injected substances. On the other hand, it is extremely improbable that the phagocytes alone produce immune body. After all that has been said we shall have to bring this production into relation with the general conditions of nutrition. The most varied cells, according to the kind of side-chains they possess and the affinities thereby brought about, are probably able to produce immune body.

Like the closely related antitoxic immunity reaction, the globulicidal and bactericidal reactions rest on a chemical process the course of which is best explained on the basis of the side-chain theory.



## V. CONTRIBUTIONS TO THE STUDY OF IMMUNITY.<sup>1</sup>

By Dr. von DUNGEN, University of Freiburg, Germany.

### A. Receptors<sup>2</sup> and the Formation of Antibodies.

ACCORDING to Ehrlich's view<sup>3</sup> the antitoxins are formed in those organs which, according to their content of receptors, have bound the toxin. Roux and Borrel<sup>4</sup> in combating to this view, have pointed out that rabbits die of tetanus following an intracerebral injection of very small doses of tetanus poison, and that therefore the brain of these animals contains no active antitoxin. Weigert<sup>5</sup> has shown that this phenomenon entirely supports Ehrlich's theory. Since the antitoxin of the central nervous system, so long as it has not been thrust off into the blood, still functionates as receptor, it must anchor the tetanus poison to the nerve cells and is therefore not at all adapted to protect these against the action of the toxophore group. Furthermore, the fact that immunized animals behave similarly proves merely that in these animals, after immunization, the ganglion cells still possess receptors. According to the side-chain theory the antitoxins present in the blood act merely by satisfying the toxins which gain access to the blood and deflect these from the organs still possessing receptors and hence still sensitive. The observations of Roux and Borrel are therefore in entire harmony with the views of Ehrlich.

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<sup>1</sup> Reprint from *Munch. med. Wochenschrift*, No. 28, 1900.

<sup>2</sup> Ehrlich and Morgenroth designate those combining groups of the protoplasmal molecule to which a foreign group, when introduced, attaches itself "RECEPTORS." See also page 24.

<sup>3</sup> *Klinisches Jahrbuch*, 1897, Vol. VI; *Werthbemessung des Diphtherie Heil-serum*, Jena, Fischer, 1897.

<sup>4</sup> *Annales de l'Institut Pasteur*, 1898.

<sup>5</sup> *Ergebnisse der allgem. Pathologie, etc.* IV. Jahrgang, über 1897

Metchnikoff<sup>1</sup> has pursued this question as to the origin of antitoxins further. Since a positive conclusion did not seem possible to him by the use of the bacterial poisons, he employed a sperm cell poison, *spermotoxin*, which can be produced by treating guinea-pigs with the testicle and epididymus of a rabbit. The use of this poison has the advantage that the organs against which it is directed can be removed from the animal without serious injury. An injection of this poison into the body of male rabbits is followed by the production of an antibody, it was merely necessary to repeat this procedure on castrated rabbits to decide the question whether the antispermotoxin is produced only by the sexual cells or also by other organs.

The results showed that the sera of rabbits which had been injected with this spermotoxin would protect rabbit spermatozoa against the action of the spermotoxin no matter whether the rabbits from whom these sera were derived had been castrated or not.

According to Metchnikoff's view, this is opposed to the chain theory, "since," as he says, "an antitoxin is produced without the presence of corresponding receptors in the organism." In this, however, Metchnikoff starts with the assumption that the spermotoxin is absolutely specific and that it acts exclusively on spermatozoa. He believes that the hæmolytic action which he has observed in the spermatozoa immune serum may be explained by assuming that with the injection of testis and epididymus red blood-cells are introduced, and that these produced a hæmolysin entirely independent of the spermotoxin. Further, he thinks that any relation of spermotoxin to other cells is excluded by the fact that in the sera of guinea-pigs which have been treated with spermatozoa the red cells suffer no greater change than they do in normal guinea-pig serum.

Having made observations in the course of my investigation of epithelial immunization, which contradict these assumptions of Metchnikoff, I feel compelled to explain my views in order to clear up the entire matter.

As I have mentioned in a previous communication<sup>2</sup> the ciliated epithelial immune serum is able, besides its specific action on ciliated epithelium, to dissolve the red blood-cells of the same animal species.

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<sup>1</sup> Annales de l'Institut Pasteur, 1900, No. 1.

<sup>2</sup> Münch. med. Wochenschrift, 1899, No. 38.

This hæmolytic property can in no way, as Metchnikoff believes, be due to the introduction of erythrocytes with the injection of the epithelial cells into the body of the guinea-pig,<sup>1</sup> which introduction would then lead to the formation of a specific hæmolysin directed against the red blood-cells. This possibility is at once excluded by the method of procedure in this experiment. For reasons of asepsis, the tracheæ employed were scrupulously cleansed with physiological salt solution and thus all traces of blood adhering to the surface were removed. The epithelium itself could not contain any erythrocytes, for it was obtained by carefully scraping the surface layer, which contains no blood-vessels. Errors due to any admixture of blood, therefore, do not enter into my experiments. Besides, such a strong hæmolytic action as is manifested by the ciliated epithelial immune serum is never produced by the injection of such small amounts of blood. In my experiments this action was greater than that following the injection of 2 cc. of cattle blood.

The strongest proof that the blood-dissolving property of the ciliated epithelial immune serum is independent of injected blood-cells is afforded by the fact that the hæmolytic immune body of this serum possesses greater affinity for the ciliated epithelium than that specifically derived by the injection of blood.

There is no doubt, therefore, that pure ciliated epithelial immune serum possesses a hæmolytic action, and that, furthermore, the hæmolysin produced by epithelial cells is different from that produced by blood-cells.

Moxter<sup>2</sup> made very similar observations on spermatozon immune serum. He found that the serum of a guinea-pig which had been treated with sheep spermatozon dissolves the blood-cells of sheep; and he demonstrated that the immune body concerned in this hæmolysis is completely bound by the spermatozon of sheep.

An absolute specificity, so that, for example, the immune body produced by means of ciliated epithelium is bound only by ciliated epithelium, that produced by means of spermatozon bound only by spermatozon, that directed against red blood-cells only by erythrocytes, without the existence of any affinities between the immune body and other cells of the same species, does not therefore obtain.

<sup>1</sup> Just as with guinea-pigs, it is possible, by injecting rabbits with tracheal epithelium of cattle, to produce a serum hæmolytic for cattle blood.

<sup>2</sup> Deutsche med. Wochenschr., 1900, No. 1.

This, of course, is readily understood by means of the side-chain theory. One could not well assume that *all* the side-chains of a certain group of cells are *entirely different* from *all* the side-chains of the rest of the cells. It is much more probable that certain groups which serve general functions of nutrition are common to the majority, if not to all, of the cells of the same animal.

When, therefore, after the injection of ciliated epithelial cells we see a hæmolytic immune body develop, we may assume that among the groups of the ciliated epithelial cell which effect the immunity, there are some which are identical with those of the red blood-cell or at least closely related to them chemically.

If this view is correct we should expect that, conversely, the immune body of an immune serum derived by treatment with blood, would be bound by ciliated epithelial cells of the same species. The facts correspond entirely with this assumption. According to my experiments, epithelial cells from the trachea of cattle are able partially to bind the blood immune body derived by treating rabbits with cattle blood. The affinity of the ciliated epithelium for the blood immune body is, however, as already mentioned, less than that for the hæmolytic ciliated epithelial immune body of the rabbit immune serum.

With this a further fact of considerable importance becomes manifest. Although the ciliated epithelial cells are destroyed by the ciliated epithelial immune body (provided sufficient complement is present), it has thus far been impossible to demonstrate any injury of these cells resulting from the binding of the active blood immune body. The epithelial cells thus differ from the red blood-cells, which are destroyed even by the antiepithelial serum. We shall not enter into an explanation of these phenomena, which point to a multiplicity of antibodies produced in response to cell material. It will suffice to point out that there is a whole series of substances which are designated as blood poisons, because they attack especially the red blood-cells while they have little or no effect on other cells.

The fact that the blood immune body when supplied with complement is bound by the ciliated epithelial cells of cattle without causing any apparent injury, proves, at least, that the phenomenon of toxic action in no way shows whether or not a toxin or toxin-containing substance has been bound by the cells. The appearance of toxic symptoms, to be sure, in the case of antitoxin-forming poisons, is proof that the poison has been bound. An absence of toxic symp-

toms may not, however, at once be ascribed to an absence of affinity between cells and the poisonous substance.

The formation of an antibody, according to the side-chain theory, follows only from the binding of the haptophore group which excites the immunity, to the corresponding side-chain, and hence is not directly dependent on the toxophore group.

As to which cells will be able to produce an antibody depends, therefore, on the possession of a receptor for the haptophore group in question. A highly toxic action of the substance bound by the cell is not at all essential, and, is in fact, often injurious, as has been emphasized especially by Knorr.<sup>1</sup> This action, as Ehrlich<sup>2</sup> has shown in his experiments on toxoids, is produced by a molecular group entirely distinct from the haptophore group and having no relation to the antitoxin.

If this law applies even to the true toxins, we shall all the more have to assume that it applies where compound substances, such as hemolysin, epitheliotoxin, or spermotoxin are concerned. In these the toxophore group is only loosely combined with the haptophore group; it is nothing but the complement, which, according to my researches,<sup>3</sup> can be bound by all kinds of cells, even independently of the immune body, and can, under certain conditions of affinity, be separated from the immune body.

We see, therefore, that the assumption by Metchnikoff, that the spermotoxin is related exclusively to the spermatozoa, is incorrect. As against it I have here shown that a toxin obtained by immunization with epithelial cells is able to destroy the red blood-cells in the same manner as a true hemolysin.

In the following short communication I can bring forward an additional instance in which the development of a hemolytic immune body results although the co-action of the red blood-cells is completely excluded. Even this demonstration proves that the assumption on which Metchnikoff based his objections to the side-chain theory is contrary to the facts. The phenomenon that even in castrated rabbits an antispermotoxin is formed is therefore readily explained according to the side-chain theory by assuming that re-

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<sup>1</sup> Munch. med. Wochenschr., 1898, Nos. 11 and 12.

<sup>2</sup> Klin. Jahrbuch, 1897, Vol. VI, and Deutsch. med. Wochenschr., 1898, No. 38.

<sup>3</sup> See page 41.

ceptors for the immune body of the spermatozoa immune serum are present not only in the organs of generation but also in other cells of the rabbit. When, in addition, we come to consider the results of these last experiments, we find that the demonstration of Metchnikoff (that even in castrated animals, in response to treatment with spermotoxin, a body is developed which prevents the action of the spermotoxin) loses all value as proof for the origin of a specific antispermotoxin.

The active spermotoxin employed by Metchnikoff is of course not a simple poison; it consists, just like a hæmolysin, of the specific immune body obtained by immunization and the complement present in all guinea-pig serum. Now it has been shown independently by Ehrlich<sup>1</sup> and Bordet<sup>2</sup> that when the complement is injected into foreign species it excites the production of an anticomplement which inhibits the action of an active immune body by taking away the complement, and that it does this without possessing any specific affinity to this immune body.

It is therefore possible that the action of the antispermotoxin obtained by Metchnikoff is to be explained thus: The injected guinea-pig serum by virtue of the complement (Bordet's alexin) which it contains, causes the production of an anticomplement serum which then renders the complement of the spermotoxin (derived from guinea-pigs) innocuous. With this idea, Bordet has examined an anti-hæmolysin, which is analogous to the antispermotoxin, and has found that the action of the anticomplement is much more pronounced than that of the anti-immune body. The formation of an anticomplement does not, of course, according to the side-chain theory, presuppose the presence of spermatozoa; for according to my experiments the complement may possess affinities for the most varied cells of the organism.

Ehrlich's theory, that the antitoxins are produced by those organs which possess chemical relations to the toxins, is therefore in no way affected by the observations of Metchnikoff.

#### B. Milk Immune Serum.

After it had been found that it is possible to produce a specific immune serum by injecting guinea-pigs with ciliated epithelium from

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<sup>1</sup> Croonian lecture, Royal Society, London, March 1900.

<sup>2</sup> *Annal. de l'Institut Pasteur*, May 1900.

the trachea of cattle it was but a step to employ epithelial secretions for the same purpose. In conjunction with this it was of considerable theoretical interest to determine in this very way whether the specific properties of cells are preserved in their secretion products.

I have therefore employed milk for immunization and have first treated guinea-pigs and rabbits with cow milk. The cow milk immune serum thus obtained is able, so far as I have been able to observe, to kill ciliated cells in the peritoneal cavity of rabbits, though in a smaller measure than the specific ciliated epithelial immune serum.

The affinities of an immune serum are readily determined when the serum, like the ciliated epithelial immune serum for example, acts also on red blood-cells, for then this can be used as a reagent. Cow milk immune serum possesses the property to dissolve cattle blood in a not inconsiderable degree. This hæmolytic action, as in the case of the blood immune serum and of the ciliated epithelial immune serum, is due not to any increased content of complement but to the presence of a specific immune body. Hence here also it was possible to compare the affinities of this immune body (for the ciliated epithelium on the one hand and for the red blood-cells on the other) with the affinities of the specific blood immune body.

The two immune sera obtained by injecting rabbits with cow milk and with cattle blood were therefore inactivated, equal quantities of normal rabbit serum to serve as complement were added to them in excess, and the mixture tested for its hæmolytic properties on cattle blood. The cow milk immune serum usually showed such a degree of action that one part of the immune serum saturated with complement was able to dissolve completely 20 parts of the customary 5% cattle blood mixture.

Corresponding to this, therefore, the much stronger hæmolytic cattle blood immune serum was diluted with inactivated normal rabbit serum or with physiological salt solution until, with an excess of complement, the hæmolytic action of the two sera on cattle blood was exactly equal.

When the two immune bodies have in this way been made entirely equal so far as the hæmolytic property is concerned, it is possible to exactly compare their chemical affinities for a particular group of cells. It is then easily demonstrated that the two hæmolytic immune bodies differ in respect to their chemical relations to other cells of the same species.

Thus if equal quantities of ciliated epithelium are added to the

two sera and the mixture centrifuged some time after, it will be found that the milk immune body has been completely abstracted from the serum, but the blood immune body only partially so. Ciliated epithelium, therefore, combines more strongly with the milk immune body than with the blood immune body.

On the other hand, the blood immune body possesses a greater affinity to the erythrocytes than does the milk immune body. Thus if equal amounts of cattle blood are added to the two inactivated immune sera (amounts which would be completely dissolved if sufficient complement were present), it will be found after a certain time that the blood immune body has been completely bound by the red blood-cells, whereas the milk immune body can still partially be demonstrated in the serum.

If one tests a number of different cow milk immune sera in this way, the results will show marked variations. My experiments were conducted on four different cow milk immune bodies which had been obtained by injecting rabbits with cow milk. Three of these showed considerably less affinity to the red blood-cells than did the specific blood immune body obtained by treatment with blood. The fourth, however, was bound by the red blood-cells in about the same degree as was the blood immune body. On the other hand, cases were observed in which the serum of rabbits after these had been injected with cow milk showed only a very slight hamolytic action, and this only on the most sensitive of the blood-cells.

All of these differences manifested themselves quite independently of the cattle blood employed in the experiment and must therefore be ascribed to differences in the immune sera themselves. Possibly they are due to variations in the kind of receptors, such as were found in a marked degree in the experiments of Ehrlich and Morgenroth on isolysins.<sup>1</sup> The strong affinity of the hamolytic milk immune body for tracheal epithelium, however, was present in all the cases examined and it did not differ materially from the chemical relationship between ciliated epithelium and its specific ciliated epithel immune body.

Hence by treatment with cow milk we obtain a hamolytic immune serum which differs from the blood immune serum, but cannot with certainty be differentiated from the ciliated epithel immune serum.

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<sup>1</sup> See page 23.



The cow milk immune serum, owing to the character of its affinities, is to be classed with the epithel immune serum.

The interesting fact to be deduced from this is that milk contains the same specific groups as the epithelial cells which produce it; and this agrees very well with histological observations according to which the protoplasm of the gland cells is itself used in the production of the milk.

After having found it possible to produce a specific epithel immune serum by injections of cow milk, it seemed to me that immunization with human milk might prove useful in the suppression of carcinoma, especially mammary carcinoma. Thus far, however, the treatment of dogs and rabbits with human milk has not yielded an immune serum hæmolytic for human blood, one corresponding to the cow milk immune serum.

## VI. STUDIES ON HÆMOLYSINS.<sup>1</sup>

### FOURTH COMMUNICATION.

By Professor Dr. P. EHRLICH and Dr. J. MORGENROTH.

THE continued thorough study of both natural hæmolysins and those produced by injections of red blood-cells leads to the conception of an extraordinary multiplicity of the substances which are either normally present in serum or which we are able at will to produce therein. That in the action of the artificially developed hæmolysins two substances are always concerned may now be regarded as a fact supported by numerous individual observations. The two substances are: (1) the specific immune body produced by immunization, and (2) a substance, usually thermolabile, contained even in normal serum, our "complement" and the "alexin" of Buchner and of Bordet. We have shown that the erythrocytes anchor the immune body in a specific manner, while they do not combine with the *isolated* complement as such. The fact that the immune body has been bound by the corresponding erythrocytes has been confirmed by von Dungern, Bordet, and Buchner. Out of a fluid containing both immune body and complement, at 0° C. the blood-cells take up only immune body, at higher temperatures both immune body and complement. We were able to explain this phenomenon only by assuming that the immune body possesses two haptophore groups, one of greater affinity, which is related to a receptor of the blood-cells and acts at 0° C., the other, of less affinity, which combines only at higher temperatures with a corresponding group of the complement.

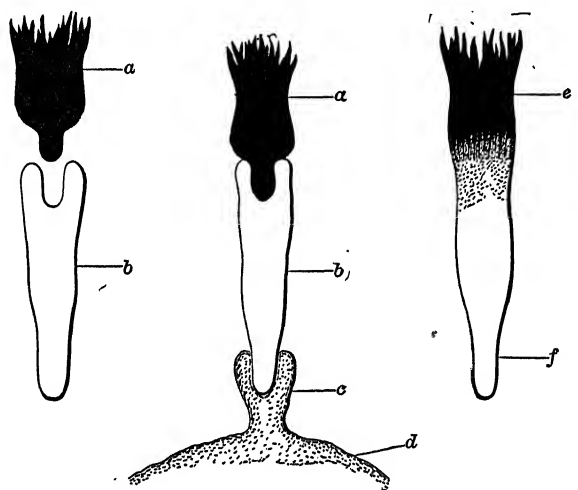
Our views can be expressed most simply by means of the following rough diagram (see figure). This will also serve to show the close relations existing between lysins and the true toxins.

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<sup>1</sup> Reprint from the Berlin. klin. Wochenschrift, 1900, No. 31.

If we bear in mind that the toxins in a restricted sense (diphtheria toxin, tetanus toxin, etc.) are characterized by two different groups, of which one is haptophore and the other toxophore, and if we express this by means of a diagram, we shall find that the analogy between toxins and hæmolysins becomes very apparent. *The active hæmolysin is seen to be nothing but a toxin consisting of two parts.* One of these parts, the immune body, corresponds to the haptophore group of the toxin, while the complement represents the toxophore:

FIG. 1.



*a*, complement; *b*, interbody (immune body); *c*, receptor; *d*, part of a cell; *e*, toxophore group of the toxin; *f*, haptophore group.

group.<sup>1</sup> In opposition to our views, Bordet assumes that the immune-body (substance sensibilatrice) in a manner not definitely stated, sensitizes the blood-cells so that certain injurious substances present in normal blood-serum (alexins) act destructively on these cells.

<sup>1</sup> This analogy becomes apparent also in heating, for the toxins as well as the hæmolysins, through the loss of the toxophore group by the one, or of the complement (which corresponds to the toxophore group) by the other, lose their specific action. On the other hand, the residues, which still possess the haptophore group, are able to excite the production of specific antibodies in the organism. In this sense, therefore, the toxoids are analogues of the immune body.

The difference between these two views is considerable. According to our views the complement (=Bordet's alexin) possesses a direct affinity, due to chemical relationship, to the immune body, while according to Bordet such a relation is excluded. Since this question concerns our scientific understanding of hæmolysins and bacteriolysins, and concerns also a basic difference affecting the practical application of the bacteriolysins, we shall have to study the subject more closely.

### I. Concerning Alexins.

Buchner, who by his thorough investigations on the bactericidal and globulicidal properties of normal sera laid the most important foundations of this subject, assumes that the serum contains certain protective bodies, alexins, which act equally on bacteria, foreign blood-cells, etc. These alexins, which are essentially of the character of proteolytic enzymes,<sup>1</sup> are of most unstable (labile) nature and lose their power by being heated to 55° C. Bordet also seems to assume the presence, in normal serum, of alexins in Buchner's sense.

According to Buchner, the serum of a given species always contains the alexin as a single definite substance. Now in our second communication we showed that the matter was much more complicated than this; that in the hæmolysins of the normal sera examined by us the action depends on the combination of two substances which correspond entirely to the two components of the hæmolysin obtained by immunization. Hence an "alexin" also consists of an interbody which withstands heating, and a complement which is generally thermolabile.<sup>2</sup> The interbody is in every respect the complete analogue of the immune body, and the only difference between these is that in one case the side-chains of the protoplasm are thrust off in the course of normal vital processes, in the other case this is due to an immunizing procedure.

Since our second communication we have been able to confirm this view by means of a large number of separate cases. Of these we shall mention only a few which serve, above all, to support the immediate consequences of our view, namely, *the multiplicity of the hæmolysins of normal serum.*

Goat serum dissolves the blood-cells of rabbits as well as those

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<sup>1</sup> Buchner, Münch. med. Wochenschrift, 1900, No. 9.

<sup>2</sup> Moxter (Centralblatt für Bacteriologie, Vol. 26) has demonstrated this also for a normal bacteriolysin.

of guinea-pigs. Heating the serum for half an hour to 55° C. causes this property to be lost, owing to the destruction of the complements. On the other hand, one frequently finds horse sera, by themselves unable to dissolve the erythrocytes of rabbits or guinea-pigs, which are able through their content of complement to complete the inactive interbody of the goat serum and make this a complete hæmolysin. According to Buchner's views, only a single alexin is concerned in hæmolysis. We therefore next studied the question whether the interbodies which act on the blood-cells of rabbits and guinea-pigs are identical. For this purpose we first determined the dose of *inactive* goat serum which, on reactivation by the addition of sufficient horse serum, was able to dissolve a certain amount on rabbit or guinea-pig blood-cells. On the basis of these data this amount of rabbit blood in physiological salt solution was mixed with the required amount of inactive goat serum and after standing a short time at room temperature the mixture was centrifuged. The result was as follows: The clear fluid mixed with additional *rabbit blood cells* and the activating horse serum showed no trace of solvent property; the red blood-cells, originally separated by centrifuging, dissolved completely under the influence of horse serum. In a parallel series of experiments the clear fluid was mixed with *guinea-pig blood*. In this, complete solution ensued.

From these experiments the conclusion follows that rabbit blood combines with an interbody present in goat serum, and does so, in fact, completely; whereas the interbody acting on guinea-pig blood is not at all fixed by the rabbit blood. By means of this elective absorption, therefore, it is positively determined that *normal goat serum contains two interbodies, one acting on rabbit blood and the other on guinea-pig blood*.

The question at once arose whether these interbodies possess a single complement in common or whether there is a special complement for each. Only after considerable labor were we able to decide this question experimentally. We were finally able to determine that in the filtration of normal goat serum through Pukall filters, the first portion (6-10 cc.) possesses a markedly different solvent power for rabbit and guinea-pig blood. We herewith reproduce an experiment of this kind.

0.15 cc. of goat-serum previous to filtration was able to dissolve completely 2 cc. of a 5% mixture of guinea-pig blood, while 0.2 cc. serum was able to dissolve the same amount of rabbit blood. After

the serum was filtered, the filtrate showed the same solvent power for guinea-pig blood, whereas the solvent power for rabbit blood had almost entirely disappeared, for 0.8 cc. effected only a trace of solution and 0.23 cc. none at all. This loss of solvent power could be due only to an absorption, by the filter, of (1) the interbody fitting the rabbit blood, or (2) the complement, or (3) both. Since, however, the solvent action of the filtrate on rabbit blood was restored by the addition of complement-containing horse serum, while the addition of interbody had no effect, it follows that the filtration had removed *only the complement*. From this fact, namely that a serum may be deprived of its complement for rabbit blood while the complement for guinea-pig blood remains, we must conclude *that there are two different complements corresponding to these two interbodies*. According to this, then, at least *four* different substances are concerned in the case in question, two different immune bodies and two complements fitting thereto. One pair of these acts on guinea-pig blood and the other on rabbit blood. According to Buchner only *one single substance*, the alexin of goat serum, would be concerned. Further details of these experiments will be published later. We should, however, like to observe that in the horse serum used for reactivating, it was possible to prove the existence of two complements. This proof, moreover, was effected in two ways, by means of filtration and by the production of anticomplements.

The following observation will show that a still greater multiplicity of normal hæmolysins can exist in the serum. In our second communication we have given a detailed description of an experiment in which a normal interbody of dog serum was caused to combine with guinea-pig blood and then reactivated by means of *guinea-pig serum*, which served to supply the complement. In this experiment the interbody contained in 0.2 cc. dog serum was bound by a certain quantity of guinea-pig blood-cells. This is the amount of dog serum which, when active, just suffices to completely dissolve the given quantity of blood. On repeating this experiment, but employing *horse serum* as complement, it was found impossible to reactivate the dose of interbody just sufficient for solution (0.2 cc.). By systematic trials, in which multiples of the dose of interbody previously used were employed, we finally determined that it required six times the amount, i.e., 1.2 cc., in order that the interbody would be completely reactivated by the horse serum. That is, the first-employed dose of the inactive dog serum, which contained just sufficient

interbody to be completely activated when the complement of guinea-pig serum was used, contained only one-sixth the amount of interbody which was completely activated when horse serum was used as complement. From this, however, it follows that all the interbody present in dog-serum and possessing specific relations to the guinea-pig blood-cells is *not of the same uniform nature*. In our case one-sixth of the interbody acting on guinea-pig blood can be reactivated by horse serum, while fully five-sixths can be reactivated by the complement of guinea-pig serum. Therefore the goat serum contains *two different interbodies for the same species of blood-cells*, and these can be positively separated by means of the difference in activation.

In our second communication, by showing the existence of a thermostabile and a thermolabile complement in the goat serum, we also proved that the complements of a given serum need not be of uniform nature. At that time we showed that the sera of two bucks treated with sheep blood-cells, as well as the sera of a number of normal goats, contained a complement which, in contrast to the other complements of the same sera (for rabbit blood and guinea-pig blood), was *not* destroyed by heating to 56° C. Buchner finds it so hard to emancipate himself from his views that he seeks to explain our observations by assuming we made a gross error in the experiment. He supposes that the sheep serum still present in the 5% mixture of sheep blood-cells, and which we disregarded, reactivated the inactive serum and led us to mistake it for a resistant complement. We were well aware of this source of error and had therefore, even in the first communication, stated that the slight amounts of sheep serum present in the blood mixture caused no disturbances whatever. How, by the way, could it be explained that these disturbances occurred only in the serum of certain animals although the method of procedure was the same? Or, that digestion of the serum with HCl, which does not injure the immune body, prevented all solution whatever?

After what has been said, we shall have to assume that in general *every serum which acts hæmolytically on various species of blood possesses a corresponding multiplicity of interbodies*, to which again different complements may fit. Against the unitarian views of Buchner and of Bordet we must uphold the view that the experimental results positively show a multiplicity of complements in normal serum. This multiplicity of the hæmolytic substances will

not be surprising if we remember that normal blood serum contains, besides the hæmolysins, a number of other active substances such as hæmagglutinins, bacterioagglutinins, antiferments, ferments, cytotoxins, etc.; and further, that from a normal serum which agglutinates several species of bacteria, the corresponding agglutinin can be isolated and abstracted by treating the serum with one of these species (Bordet); and that the same holds true for hæmagglutinins (Malkoff). We shall quite naturally come to the conclusion that, under normal conditions of the cell's nutrition, a large number of simple or complex side-chains are constantly thrust off which then, either alone or in conjunction with complements similarly thrust off, exert specific actions. Hence normal serum contains an enormous number of such substances. To these, in general, we give the name *haptins*.

When therefore Buchner, in opposition to our views, believes that the assumption of these different substances seems unreasonable, we must emphasize that our conclusions are not the result of speculation, but simply the necessary consequences of observations which are not to be harmonized with the assumption of a single simple alexin. It will be evident also why we have completely dropped the term alexin used by Buchner. In our investigations, in all the cases closely analyzed, we never found a simple substance (Buchner's alexin), but always a complex hæmolysin consisting of interbody and complement. This hæmolysin, as already emphasized, completely corresponds in its properties to the hæmolysins developed through immunization. We shall therefore have to assume that also in their development the normal hæmolysins correspond exactly to the artificial hæmolysins.

In regard to the latter, von Dungern has already shown, by demonstrating a great disproportion between immune body and complement, that these two substances are produced quite independently of one another, and that they therefore probably originate in different cell domains. von Dungern also showed that in the extensive formation of new immune body which occurred when rabbits were treated with cattle blood-cells, the corresponding complement was not in the least increased. We ourselves have often noted an analogous independence of the two components in a number of normal hæmolysins. One of us will discuss this at length in a subsequent paper. One interesting fact, however, we shall mention here.



If rabbits are poisoned with a dose of phosphorus, of which they die on the third day, and if the serum of the animal is collected on the second day, it will be found that the serum has lost the property, previously possessed, to dissolve guinea-pig blood. This inactive serum can be activated by the addition of a sufficient amount of guinea-pig serum. It behaves, therefore, like a serum which has been inactivated by heating to 55°C., i.e. it has been deprived of its complement. It is probable that the phosphorus has acted especially on certain cell domains which furnish the complements in question.

## II. Concerning Anticomplements.

In accordance with the views already discussed in detail, we assume that the hemolytic action is due to this, that the interbody (immune body) and complement unite to form the complex hemolysin. We can understand such relations only when we regard them stereochemically and must therefore assume that the complement possesses a haptophore group which finds in the interbody a receptor group into which it exactly fits. With this conception, however, the relations existing between interbody and complement at once assume a strictly specific character, i.e., the interbody and complement become strongly specifically related. As a result of combining experiments we have already<sup>1</sup> attacked the view of Bordet, that the immune body merely sensitizes the red blood-cells and that as a result of this sensitization the alexins, which otherwise are unable to attack the blood-cells, now have access to them. That the "substance sensibilisatrice" breaks the way for the alexins is a coarse mechanical conception hardly comprehensible when viewed chemically or biologically. If one sought to explain Bordet's view chemically, one would have to assume that the nature of the sensitization is this, that under the influence of the sensitizor a whole series of groups are developed in the protoplasm of the red blood-cells which are able to bind the various complements. Such an assumption, however, lacks every element of probability. Bordet<sup>2</sup> himself arrives at a contradiction when on the one hand he assumes a direct action of the complements on the red blood-cells and on the other is forced to admit that certain relations exist between interbody and complement

<sup>1</sup> See our second communication.

<sup>2</sup> Bordet, *Annales de l'Institut Pasteur*, May 1900.

(certains rapports convenables). It would be difficult to express these relations in a form chemically comprehensible.

Based on the conception of strictly specific relations, such as follows from our theory, the study of these complements acquires a high practical value. Dönitz<sup>1</sup> has already called attention to the great importance for the therapy of infectious diseases of finding sources yielding sufficient complement. von Dungern<sup>2</sup> has furthermore shown that body cells are able to bind certain complements and that therefore a completed bacteriolysin derived from a certain animal species can, when it is injected into another organism, entirely lose its complement and so become inactive.

In the Croonian lecture (March 22, 1900), Ehrlich pointed out that the bacteriolysins and hæmolysins (interbody+complement) possess three haptophore groups, of which two are on the interbody and one on the complement. It is conceivable that for each of these groups there is a corresponding antigroup which binds the haptophore concerned and so inhibits the action of the lysin. For each lysin therefore three antibodies are possible, the action of any one of which is able to put the lysin out of action. At that time Ehrlich called particular attention to the important rôle of one of these antibodies, namely, the one which fits into the haptophore group of the complement and so prevents this from combining with the interbody (immune body). He stated further that together with Morgenroth he had succeeded in the experimental production of such anticomplements by means of immunization.<sup>3</sup>

Our observations in this direction were made on the serum of a goat which for a long time had been injected with large amounts of horse serum. Horse serum was used because our extended observations had shown that this constitutes a particularly rich source of most varied complements, and because it was therefore to be expected that a plentiful amount of anticomplements would be obtained. This expectation was fully realized, and we have come to know a large number of interbodies of different origin which can be reactivated by the complements for different varieties of blood contained in horse serum. As an example the following combinations may be mentioned: Rabbit blood—inactive dog serum; guinea-

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<sup>1</sup> Dönitz, *Klinisches Jahrbuch*, Vol. 7, 1899.

<sup>2</sup> See page 36.

<sup>3</sup> In the meantime Bordet (loc. cit.) independently has also produced anticomplements by means of immunization.

pig blood—inactive goat serum; sheep blood—inactive dog serum; sheep blood and inactive serum of goats treated with sheep blood. In all these cases we have been able to determine that the reactivating action of the horse serum can be prevented by the addition of small amounts of anticomplement serum (previously inactivated).

In one case a very minute analysis of this action was made. The factors in this case were rabbit blood and an interbody acting on this, present in normal goat serum and obtained by heating the serum to 56° C. The rabbit erythrocytes were first treated with considerable amounts of this interbody and the excess of interbody was then separated by centrifuging the mixture and pouring off the clear fluid. The erythrocytes thus loaded with interbody were next digested with large amounts of the inactive anticomplement serum and this likewise separated by centrifuging. The sedimented blood-cells thus obtained dissolved completely on the addition of horse serum. The same result was attained when the process just described was performed in one act instead of in two; i.e., by mixing the goat serum containing the interbody with the anticomplement serum *before* the addition of the blood-cells.

From this it follows that the antibody stands in relation neither to the blood-cells themselves nor to the interbody. Even in the presence of the antibody the interbody is anchored in normal fashion by the erythrocytes, and is furthermore not disturbed in its receptive property for the complement. The antibody therefore has no relation to either of the two haptophore groups of the interbody, and it can only act by influencing the complement.

The complement, however, according to our view, also possesses two groups: one, a haptophore group, and a second which, in order to express the analogy to the enzymes and toxins, we shall term the *zymotoxic* group. Hence it still remained to determine into which of these two groups the anticomplement fits. In either case, though of course by a different mechanism, the action of the complement would be inhibited; in one case by preventing the combination of complement and interbody, in the other by preventing the zymotoxic action.

If we assume that the anticomplement combines with the zymotoxic group, then the haptophore group of the complement will remain free and must still be able to combine with the corresponding group of the interbody. It would be expected, then, that the haptophore group would combine with the interbody and "plug," so to speak,

the binding group of the latter against any further complement. If, on the contrary, the anticomplement with the haptophore group of the complement, the is left free and must therefore still be capable of reactivating experimental solution of this question was very easy. Erythrocytes, loaded with interbody, were subjected to the mixture of complement and anticomplement which had been neutralized to complete inactivity. After centrifuging it was found that the blood-cells dissolved readily on the further addition of complement. Solution also occurs if a small amount of complement in excess is added to the exactly balanced mixture of complement and anticomplement. These experiments indicate that the anticomplement *acts by fitting into the haptophore group of the complement, thus side-tracking this group.*

We have also convinced ourselves that it is possible to produce anticomplements not only with horse serum but also with sera of other animals, such as the sera of goats, dogs, cattle, rabbits, and so on, by injecting the serum into foreign species. In these experiments the choice of animals employed for purposes of immunization is of an important rôle. For example, a rabbit treated with complement very readily yields an anticomplement, whereas when a horse is similarly injected no anticomplement (at least in the amount examined by us) could be demonstrated. So far as we are able to determine, the protection afforded by the anticomplement is common to all the species of blood-cells on which the serum used for immunization exerts its action. Since the sera in question, so far as their action is concerned, contain a plurality of complements, a complementary serum must contain a whole series of anticomplements which correspond to the different complements of the immunizing serum. Perhaps this polyvalence of the complementary serum accounts for the phenomenon that certain sera produced by means of a particular blood serum can inhibit the injurious action of many other kinds of blood serum. These facts indicate that this interchange of protection is due to the presence in the two sera of a certain number of complements. In fact there seem to be cases in which certain sera have the *majority* of their complements similar. Such a high probability is that of the goat and the sheep, as is evident from the fact that in the reactivating action goat serum can be replaced by sheep serum and vice versa. This at least

all the cases observed by us. Still more convincing, however, is the fact that neither the injection of a sheep with goat serum nor of a goat with sheep serum results in the production of anticomplements. All experiences indicate that the complements normally present in the serum of a certain species of animal are *not* able to excite the formation of anticomplements in such an animal's *own* body. Perhaps this may be explained thus, that the relation between complement and complementophile group is extremely slight (as was shown by the binding experiments previously described by us) and that therefore *one* of the conditions necessary for the thrusting off—a permanent and firm union with the receptor—is not in this case fulfilled.

We realize that we have been able here merely to point out some of the principles applying to this subject. Their closer analysis encounters extraordinary difficulties in consequence of one of the facts demonstrated by us, namely, the multiplicity of interbodies, complements, and anticomplements. Thus far these difficulties have been overcome in only a few favorable instances.

### III. One of Bordet's Objections Controverted.

Bordet, in his most recent work (*loc. cit.*) has described the following interesting experiment, by means of which he believes to prove that our views concerning the mechanism of hæmolysis are incorrect. As hæmolysin, Bordet employed the serum of guinea-pigs after these had been treated with rabbit blood. This then possessed a high degree of solvent power for rabbit blood. If this hæmolysin is inactivated by heating, it is possible to restore the hæmolytic action, as well by the addition of normal guinea-pig serum as by that of normal rabbit serum. These two sera, therefore, contain complements (alexins) which make the reactivation possible. Bordet now sought to discover whether the "alexin" of rabbits is identical with that of guinea-pigs. For this purpose he treated rabbits with the serum of the immunized guinea-pigs and obtained an antiserum which, while it contained a small amount of anti-immune body, contained considerable anticomplement. He then determined that this "antialexin" acted only against the "alexin" of the guinea-pig and not at all against that of rabbits and some other animals. At the same time a certain degree of action against the complement of pigeon serum was noted, so that this antiserum was not absolutely specific. From this Bordet concludes that his theory of sensitization must be correct, namely, that the various alexins derived from

different species act *directly* injuriously on the sensitized blood-cells. Against each of these alexins an antialexin exists which protects the sensitized blood-cells against just this particular alexin.

It cannot be denied that at first sight this experiment appears to speak strongly in favor of Bordet's theory. If one assumes, Bordet of course does, that in the immune serum produced by *one single immune body* comes into play, then since this can be reactivated as well by rabbit serum as by guinea-pig serum, the complement contained in these two species of sera must, according to our theory, possess the same haptophore group. If this were the case, however, the *same anticomplement* should protect against *both complements*, and this it does not do.

We have therefore subjected Bordet's experiment to an *exhaustive* reexamination and have been able to determine that an exhaustive quantitative analysis presents the experiment in an entirely different light. A hæmolytic serum was produced by treating guinea-pig blood with rabbit blood. A preliminary trial of this serum showed that when inactivated it could be reactivated in large amounts as well by guinea-pig serum as by rabbit serum. The anticomplement derived from other rabbits by treatment with normal guinea-pig serum,<sup>1</sup> was able in the inactive state to completely inhibit the reactivation with guinea-pig serum, although the same anticomplement serum in its active state reactivated the inactive immune body.

We next proceeded to examine these facts quantitatively and found that the simple solvent dose of the serum for 0.5 cc. of a 5% rabbit-blood mixture amounted to 0.075 cc. Then we tried von Dungern's experiment (*loc. cit.*) to increase this action, by adding to the native immune serum normal guinea-pig serum in amounts so small that they did not themselves exert any solvent action. We found that the full solvent dose had thus been decreased to 0.025 cc. This proved, as in von Dungern's case, that in the immunization a large excess of free immune body was present which could not nearly be satisfied by the amount of complement normally present. Now we could expect that this same increase in power would be effected by the addition of rabbit serum, *but we found instead that rabbit serum even in large amounts did not produce any increase whatever.*

According to Bordet's view such a deviation is absolutely incomprehensible, and this led us to pursue the case further. We first

<sup>1</sup> In contrast to Bordet we chose *normal* guinea-pig serum for immunization in order to avoid the disturbing action of an immune body.

inactivated the immune serum and determined the minimal amount of the *inactive* serum which would cause complete solution in the presence of (1) normal rabbit serum, or (2) of guinea-pig serum. We found that it required 0.25 cc. of the inactive immune serum to effect complete solution of the given amount of rabbit blood when rabbit complement was employed, whereas only 0.025 cc. of the immune serum was required when guinea-pig complement was employed.

This result, however, cannot be harmonized with Bordet's theory of sensitization. According to his view one would expect that a blood-cell which is sensitized by the presence of the immune body is subject equally to the action of various alexins. In both cases the *same amount* of immune body should then suffice to make the blood-cells sensitive to the alexins (complements). As a matter of fact, however, it requires ten times as much in the one case as in the other. If one desired to hold to Bordet's theory one might possibly say that it requires ten times as strong a sensitization with the same immune body in order to make the cells sensitive to the alexin of rabbit serum.

If this highly complicated assumption were correct, the relation as above determined, 1 : 10, should represent a constant ratio. Owing to a lack of animal material we were unable to study this question of constant ratio on the example selected by Bordet. However, in an analogous series of cases for which we had abundant material, we were able to pursue this question further.

We made use of a goat which had been treated with sheep blood and whose serum therefore dissolved sheep blood-cells. The inactivated serum of this goat could be reactivated by two complements, that of normal goat serum and that of horse serum. The anticomplement obtained by treating a goat with horse serum inhibited, even in small amounts, the action of the horse complement; whereas its action on the goat complement was so slight as to be practically negligible. The conditions here, therefore, are exactly the same as in the case described by Bordet.

In the beginning of the observations it was found that 1 cc. of a 5% mixture of sheep-blood, mixed with normal horse serum to serve as complement, was completely dissolved on the addition of 0.35 cc. immune body (inactivated immune serum); whereas when normal goat serum was used as complement only 0.025 cc. of the immune body was required. This corresponds to a ratio of 14 : 1. On repeating the test a week later with serum freshly drawn from the immunized goat we found that the constituents which were



reactivated by horse serum were unchanged (0.35), but that it required considerably more immune body when goat serum was used for reactivation than it had before, namely, 0.1 cc. This corresponds to a ratio of 3.5 : 1 as compared to the former ratio of 14 : 1. This shows that a constant ratio does not as a matter of fact exist. We must rather assume, as we did for a *normal* hæmolytic serum, that two entirely independent immune bodies, A and B, are present in the immune serum and that these differ in the ratio of their quantities and in the manner in which they are reactivated. The amount of immune body A contained in the immune serum has remained constant, while B after a short time has considerably decreased (to one quarter). This divergence would in fact indicate that the two immune bodies are formed independently of each other.

We have thus demonstrated that in the phenomenon observed by Bordet not a single immune body, but two different ones, come into play, one of which is related to a complement found only in guinea-pig serum, while the other is related to a complement found in rabbit serum. Through this demonstration Bordet's objection loses all its force and his experiment becomes in fact *a new argument for our theory*.

The occurrence of different immune bodies in a hæmolytic serum obtained by immunizing with red blood-cells is not at all surprising in view of our experiments on isolysins described in our third communication. We have obtained a whole series of *different* isolysins by injecting goats with goat blood. At present they number twelve. In the red blood-cells not merely a single group but a large number of different groups must be considered, which, provided there are fitting receptors, can produce a corresponding series of immune bodies. All of these immune bodies again will be anchored by the blood-cells employed in immunization. We may assume that when an animal species A is immunized with blood-cells of species B a hæmolytic serum will be produced which contains a great host of immune bodies. These immune bodies in their entirety are anchored by the blood-cells of species A.

We are convinced that the duality found by us in the two cases examined is much below the actuality, and that thorough, though to be sure arduous, studies will succeed in discovering a multiplicity heretofore unexpected. For the present, however, this duality of the immune body should suffice to controvert the objections made by Bordet from the unitarian standpoint.



## VII. STUDIES ON HÆMOLYSINS.<sup>1</sup>

### FIFTH COMMUNICATION.

By Professor Dr. P. EHRLICH and Dr. J. MORGENROTH.

IN the few years since its formulation the side-chain theory has exercised a marked influence on the direction of the investigations in immunity. The subject of toxins and antitoxins has to a certain extent been concluded, at least for the present. Several objections raised by Roux and Borrel<sup>2</sup> in connection with their splendid work on cerebral tetanus, as well as those made by Metchnikoff<sup>2</sup> and Marie,<sup>2</sup> rested on a misconception of the theory, and the facts on which these are based serve rather as a complete confirmation of the theory.<sup>3</sup> The attempt of Pohl<sup>4</sup> to place the doctrine of antitoxins purely on the basis of inorganic chemistry has been completely controverted by Bashford.<sup>5</sup>

Thus the facts proved themselves thoroughly in harmony with the theory, and the latter furthermore proved its inventive value in many directions. It was but natural that the side-chain theory originally formulated for the antitoxins, if it had any general biological significance at all, should also include the complicated phenomena of immunity which result from the introduction of bacteria or tissue-cells. Hence we began two years ago to investigate experimentally the applicability of the doctrines resulting from this theory to the specific hæmolysins obtained by immunization, which had been discovered by Bordet a short time previously. These studies

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<sup>1</sup> Reprint from the *Berliner klin. Wochenschrift*, 1901, No. 10.

<sup>2</sup> *Annales de l'Institut Pasteur*, 1898.

<sup>3</sup> See Weigert, Lubarsch's *Ergebnisse der Pathologie*, 1897; also Levaditi *Press médicale*, 1900, No. 95.

<sup>4</sup> *Arch. internat. de Pharmacodyn.*, 1900.

<sup>5</sup> *Arch. internat. de Pharmacodyn. et Therapie*, Vol. VIII, fasc. I and II, 1901.

served to demonstrate the complete harmony of the theory with the facts on this subject. Furthermore after overcoming considerable experimental difficulties we succeeded in demonstrating the same behavior for the hæmolysins of *normal* serum and thus brought these also under the laws of the side-chain theory. Reexaminations from various directions confirmed the correctness of our fundamental experiments and we may say that at present the majority of workers in this field, partly as a result of their own experiments, have accepted our views and regard the side-chain theory as a justified hypothesis which best explains most of the phenomena thus far observed in the subject of immunity. Since this in part concerns processes in which the animal organism acts with all its highly complicated conditions, it is no wonder that now and then a fact has appeared in the course of the investigations which at first seemed to be irreconcilable with the theory. The latter, however, is in no way injured thereby, for the solution of such apparent contradictions results in a deeper understanding of the subject and makes for progress. An instructive example of this was recently afforded in physical chemistry. As is well known, several at first inexplicable contradictions to van't Hoff's theory of solutions, resulting from certain deviations in osmotic tension, found their explanation in the theory of electrolytic dissociation of Arrhenius, and this theory served to again obtain general acceptance for the theory of solutions itself. We have therefore endeavored to analyze carefully the objections urged against our views by high authorities.

The objection raised by Metchnikoff<sup>1</sup> against the specific formation of the toxins was based on the fact that even castrated rabbits yield an antispermotoxin. In a recent study<sup>2</sup> from the laboratory of Metchnikoff, this objection is withdrawn. It was found that in this antispermotoxin an anticomplement is principally concerned and not an anti-immune body, for it was produced even by treatment with normal serum.<sup>3</sup> It is therefore especially gratifying that Metchnikoff also has recently accepted our view that the complement is anchored to the immune body by means of the latter's complementophile group.

An important objection made by Bordet<sup>4</sup> based on some extremely

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<sup>1</sup> Annales de l'Institut Pasteur, 1900, No. 1.

<sup>2</sup> Ibid., No. 9.

<sup>3</sup> See von Dungern, page 47.

<sup>4</sup> Annales de l'Institut Pasteur, 1900, No. 5.

testing experiments, by which he believed to refute our theory of the mechanism of hæmolysis, has been discussed by us in our last communication<sup>1</sup> and controverted by means of extended quantitative experiments.

It is necessary, however, once more to thoroughly discuss the binding of immune body to the erythrocyte, for on this point the views seem not at all clear, because the purely chemical conception is held by some authors or is regarded as unimportant.

### The Manner in which the Immune Body Combines with the Erythrocytes.

In our first communication we had already shown that the erythrocytes as such behave quite differently toward the two components which effect hæmolysis. The blood-cells abstract the immune body from its medium with great avidity, whereas they do not take up the least trace of complement. When loaded with immune body, however, they are able to anchor the complement also. From this we have concluded primarily that the immune body possesses two binding groups of different affinity, of which the *one* combines with the responding group, the *receptor* of the blood-cell; the *other* combines with the complement. But according to our view these combinations are pure chemical phenomena proceeding between immune body and blood-cells and between immune body and complement. The function of the immune body can be elucidated by means of a chemical example, that for instance afforded by the behavior of benzaldehyd. Through its diazo group this substance can combine with a series of bodies, especially with amines, phenols, ketodien groups, whilst the aldehyd group on its part can effect a series of syntheses—e.g. with hydrazins, hydrocyanic acid, etc. It becomes easy by means of diazobenzaldehyd to effect a combination between substances which by themselves do not combine, phenol and hydrocyanic acid. Such a combination includes *both* substances. In order to make the comparison still closer let us imagine certain constituents of the living cell, say by means of an aromatic group, are able to unite with the diazo combination. In this case it follows that by means of the aldehyd group of the diazobenzaldehyd a second highly toxic nucleus—e.g. that of hydrocyanic acid—can be joined to the combination in such fashion that the protoplasmic molecule is now subjected to the action of the strongly

<sup>1</sup> See page 56.

acting nitril group. In this schematic example the diazo group which fits directly into the protoplasm would correspond to the haptophore group of the immune body which fits into the receptor of the blood-cells; the aldehyd remnant would correspond to the complementophile group of the immune body. The complement, which as we know possesses toxic properties, would then be compared to the hydrocyanic acid.<sup>1</sup>

The facts described by us have been confirmed from various sides (v. Dungern, Buchner, Bordet) by experiments on blood-cells. Bordet<sup>2</sup> and also Nolf<sup>3</sup> showed that the *stromata* of the blood-cells, which represent the protoplasm, effect the anchoring of the immune body, while the hæmoglobin, which is to be regarded as paraplasm, takes no part whatever in this binding. This fact corresponds entirely to the views expressed by Ehrlich in an earlier study on blood-cell poisons.<sup>4</sup> Furthermore, it has been shown by von Dungern<sup>5</sup> that the power of the blood-cells to excite a specific hæmolysin by immunization can be entirely inhibited by completely loading the receptors of the blood-cells with the immune body in question. These additional facts were well fitted to still further support the chemical conception of these processes.

Now, however, Bordet has described an experiment which he believes shows that the fixation of the immune body is not a chemical process in the strict sense, but that this phenomenon is to be classed rather with surface attraction and similar actions, and that it is completely analogous to staining processes. These views are also shared by Nolf<sup>6</sup> and Nicolle.<sup>7</sup>

Bordet's experiment in the main is as follows: By treating a guinea-pig with rabbit blood a hæmolytic serum is obtained specific for rabbit blood-cells. A certain amount of the serum dissolves an absolutely definite amount of rabbit blood-cells if all the cells are added to the serum at once. If, however, to the same amount of serum

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<sup>1</sup> One could designate substances which, like the immune bodies, are supplied with two different combining groups as *amboceptors*. This name would indicate the double binding function as well as the fact that they correspond to thrust-off receptors.

<sup>2</sup> Loc. cit.

<sup>3</sup> Annal. de l'Institut Pasteur, 1900.

<sup>4</sup> Charité Annalen, Vol. X.

<sup>5</sup> See page 36.

<sup>6</sup> Loc. cit.

<sup>7</sup> Revue générale des Matières Colorantes, 1900, Nos. 43 and 44.

only one-half the amount of blood-cells is first added, sufficient time allowed for these to completely dissolve and the second half of the blood-cells added, it will be found that these are no longer dissolved. It appears, therefore, as though the blood-cells were capable of combining with double the amount of immune body necessary for their solution. In order to explain this result Bordet describes the following staining experiment: If one dissolves methyl violet in water, it is possible, by means of a strip of filter-paper dipped into the solution, to abstract all the coloring-matter from the solution. The strip will assume a color of very definite intensity. If, however, the strip is divided into several smaller strips and these are dipped into the fluid *one after the other*, the first strip will assume a considerably deeper color, whereas the strips last introduced will be unable to abstract any color from the now colorless fluid. From this Bordet draws the following conclusion:

"On peut admettre, par comparaison, que les premiers globules introduits dans l'hémotoxine sont déjà susceptibles de perdre leur hémoglobine lorsqu'ils ne sont encore que "faiblement teints" par les principes actifs, mais qu'ultérieurement ils peuvent absorber une dose beaucoup plus grande de ces substances, épuiser ainsi le sérum et empêcher la destruction de nouveaux globules introduits dans la suite."

Phenomena such as those here described have long manifested themselves in our experiments on the binding of the immune body by the erythrocytes although these experiments were of somewhat different form. But before we proceed to discuss these results and our conclusions, we should like to describe the facts observed by us.

In order to determine the combining ability of the erythrocytes for an immune body, especially when quantitatively accurate results are desired, it is best to proceed as follows: The immune body (hæmolysin heated to 56° C.) is added to the red blood-cells and, after a certain time, the mixture is centrifuged. The clear fluid so obtained is tested for free immune body by adding an excess of complement and allowing this mixture to act on the same quantity of fresh blood-cells. If one proceeds in this manner in a large series of cases, employing varying multiples of the solvent dose of immune body, it is possible to determine accurately the combining power of the cells. The following experiment will very readily make this clear.

The immune body was present in the serum of a sheep which had been treated with dog blood. When this serum was inactivated

by heating to 56° C., it could be reactivated either with the complement of sheep serum or of goat serum. To begin, the exact quantity of immune body was determined which would just completely dissolve 2 cc. of a 5% mixture of dog blood-cells when sufficient complement was present. This dose was found to be 0.15 cc. To a number of separate portions of blood mixture (each of 2 cc.) multiples of this dose were then added, thus, 1, 1½, 1½, 1½, 2, 2½, 3 times the solvent dose, and the mixtures kept at room temperature for an hour and frequently shaken. Since the complement was absent, hæmolysis could not occur. After centrifuging, the clear fluid, which had the appearance of water, was again mixed with the corresponding amount of blood (0.1 cc. of undiluted blood) and with complement.<sup>1</sup> It was found that even the last trace of the single solvent dose had disappeared from the fluid; whereas in the case where double the dose had been added, the fluid still contained just a solvent dose, i.e., it completely dissolved the freshly added blood-cells. In this case, therefore, the blood-cells were able to combine with *only a single dose of the immune body*.

This, however, is not at all the general rule, for by extending our experiments to other cases we found that there is a very large variability in this binding of the immune body, and that frequently a larger or smaller multiple of the solvent dose is bound. The following case will illustrate the extreme in the other direction, *in which almost a hundred times the solvent dose of immune body was taken up by the blood-cells*. A rabbit had been treated with goat blood, and its serum therefore contained an immune body fitting to goat blood. Normal guinea-pig serum served as complement and 0.2 cc. represented considerably more than sufficient for 2 cc. of the goat blood mixture. When this amount of complement was employed, the solvent dose of the immune body for 2 cc. of the blood mixture amounted to 0.008 cc. On allowing 0.48 cc. (sixty times the solvent dose) to act on the blood-cells in the manner previously described, and then centrifuging, it was found that the clear fluid did not contain even a trace of immune body. When eighty times the dose was employed the clear fluid showed a very faint solvent action, corresponding to about ⅙ to ¼ of a solvent dose. Not until one hundred times the dose

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<sup>1</sup> As a counter test the blood-cells separated by centrifuge were mixed with salt solution and with the complement. Those specimens in which just the solvent dose (0.15 cc.) of the immune body or more was present, dissolved completely.

was employed did the centrifuged fluid contain a full solvent dose and effect complete solution. Hence out of one hundred solvent doses about ninety-nine had been bound by the blood-cells, for only about one solvent dose of immune body remained in the fluid. By means of parallel experiments we have found that one hour's contact of immune body with blood-cells results in the maximum amount of binding, for the experiments at 45° C. and room temperature yielded results exactly alike. Between the extremes represented by these two experiments a great variety of figures was obtained.

The significance of these experiments offers no difficulties from the point of view of the side-chain theory. The facts are readily understood when we stop to consider the peculiarities of the receptor apparatus of the blood-cells. As a result of our previous experiments on the isolysins of goats we assume that a given blood-cell contains a large number of different types of receptors which in general fit to different immune bodies and hemotoxins. Referring the reader to an exhaustive study by Ehrlich,<sup>1</sup> we shall content ourselves here by remarking that certain kinds of receptors may be present in the blood-cell in great excess. This excess cannot only be demonstrated, but, by means of the method just described, can be exactly measured. Entirely analogous conditions arise under other circumstances. The interesting fact discovered by Wassermann, that the central nervous system of various animals binds much more tetanus poison *in vitro* than is necessary to fatally poison the animal, is probably due to such an excess of receptors for tetanus poison.

From this point of view the experiments above mentioned are easily explained without departing from the side-chain theory. Thus, let us assume that with a certain poison *a* it is necessary that *x* *a*-receptors are bound in order that a blood-cell be completely dissolved, and let us further assume that the blood-cell possesses a much greater number, say 2*x* *a*-receptors. When Bordet's experiment is now carried out, the conditions arising will be exactly those described by Bordet. It is at once apparent that the red blood-cell in this case will combine with just twice the amount of poison necessary for its solution. If therefore double the solvent dose of immune body is added to a given amount of such blood-cells, the entire receptor

<sup>1</sup> *Specielle Pathologie und Therapie*, edited by Nothnagel, Vol. VIII, section 3, pages 163-184.



system of these cells will be occupied. On adding now an equal portion of fresh blood, the latter will fail to find any free immune body and cannot therefore be attacked.

Such phenomena are exceedingly plentiful in chemistry, and it may pay us to glance at some of them. Naphthalin, as is well known, consists of two benzole nuclei joined together. When, now, a salt-forming group, *hydroxyl* or *amido* group, is introduced into each of the two benzole nuclei, the heteronuclear substitution products, e.g., *dioxynaphthalin*, *amidonaphthol*, and *naphthylenediamine*, or their sulfo acids, will be able to combine with either one or with two molecules of a *diazo* combination. When *two* molecules of *dioxynaphthalin* are mixed with *two* molecules of *diazobenzol*, the result is exclusively the *mono-azo* combination; when however *two* molecules of *diazobenzol* are added to *one* molecule of *dioxynaphthalin*, the result is the *diazo* combination. If an additional molecule of *dioxynaphthalin* is added to the finished *diazo* combination, this molecule will be unable to dissociate the latter, and the two substances, the *diazo* combination and the unchanged *dioxynaphthalin*, will exist side by side. This example, to which others, such as the esterification of dibasic acids, the methylation of anilin with iodomethyl, could easily be added, corresponds entirely to the relations between immune body and erythrocytes as described by Bordet.

It may at once be admitted that where the binding of small multiples of the immune body is concerned, it is very natural to think of a mechanical absorption due to the degree of concentration; and that therefore the circumstances in Bordet's case, in which the binding was merely doubled, justified the comparison with staining processes. The cases examined by us, however, in which at one time just the solvent dose of immune body, at another an extraordinarily large multiple of the dose was bound, weigh heavily against this assumption.

Our decision, however, is especially determined by certain general considerations. Thus, charcoal, the type of surface-attractive agents attracts thousands of substances of the most varied kind. A dye can stain a large number of different substances, as is shown in every stained microscopic preparation. In marked contrast to this is the *specificity* of the numerous antibodies, which primarily are always directed against the exciting bacterial or other cell species.

In the cases in which apparent deviations from this rule were



noted, exact investigation has shown<sup>1</sup> that these are due to the presence of one and the same receptor group in various elements. Thus we have shown that the isolysins produced by injecting goats with goat blood-cells act also on sheep blood-cells. We have further shown that these sheep blood-cells possess certain kinds of receptors which bind the goat lysin just as the receptors which are present in the goat blood-cells do. We produced the strongest proof for this community of receptors by means of crossed immunization, for we succeeded in producing a typical isolysin by injecting goats with sheep blood.

Since all experiences, therefore, lead us to assume that each particular complex produces just the specific antibody, and since this agrees exceedingly well with the assumption of a chemical union, it would be a distinct backward step to adopt so vague a conception as that of mechanical surface attraction.

Were we to assume that the immune body enters the cell merely mechanically, it would be necessary to drop the entire unity of the immunization phenomena which follows from the side-chain theory. It is probably quite generally conceded that the antitoxin acts on the toxin in a purely chemical manner. Hence so far as *dissolved* substances developed by the immunity reaction are concerned, the *chemical* conception applies. Why then should this chemical action suddenly cease when the substances instead of being in solution are present within the cell, and a new principle be assumed for this case? This leads to the contradiction that in one case (when combining with the erythrocytes) the immune body is bound, specifically to be sure, but *mechanically*, while in the other case (when anchored to an artificially produced anti-immune body in solution) it is bound specifically but *chemically*.

These considerations, and they could readily be greatly extended, will suffice to show that the above-mentioned experiments are not at all capable of shaking the side-chain theory, for by it alone is a single uniform conception of the phenomena of immunity rendered possible.

## II. Concerning Complementoids.

The complements, which effect the activation of the normal immune bodies and of those produced by immunization (amboceptors) do not possess great theoretical or practical importance in the study

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<sup>1</sup> See Third Communication, page 23.

of immunity. They seem to play an important rôle in the normal processes of cell nutrition. As a result of experiments already described we must assume that in the blood serum of a particular animal species not merely a single complement exists but a large number of different complements. It is understood, of course, that not all the complements occurring in a large number of species differ from one another. On the contrary it is to be regarded as certain that particular types find a wide distribution extending over several animal species. This explains why, for example, a hæmolytic or bacteriolytic immune body can be reactivated by the sera of *different* animal species.

We have previously explained that a complement is to be conceived as possessing *two* characteristic groups, a haptophore group which fits into the complementophile group of the immune body, and a zymotoxic group which is the actual carrier of the specific action. A complement therefore, to a certain extent, corresponds to a toxin, which possesses a haptophore and a toxophore group. Hence by the immunization of suitable animals it is easy to obtain anti-complements whose behavior corresponds exactly to that of anti-toxins. For example, if a goat or rabbit is injected with horse serum, an anticomplement will be formed which is able to specifically inhibit the action of the complement contained in horse serum. We have already shown<sup>1</sup> that this is due to a deflection of the complement.

We have now tried to follow this analogy (between complements and toxins) further. We take it for granted that it is generally known that toxins, either through spontaneous changes or through the action of chemical agents, become modified into *toxoids*, whose distinguishing character is that they no longer possess a toxophore group although the haptophore group remains. These toxoids, then, are relatively non-toxic substances which are nevertheless able to cause the formation of antitoxins in the animal body. Now we know that the zymotoxic group is extremely sensitive to the most varied influences; hence the attempt to study modifications of the complements analogous to the toxoids seemed to promise favorable results. Such modified complements would then be designated *complementoids*. The first step was to see whether the well-known inactivation of a serum by heating to 56° C. completely destroyed

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<sup>1</sup> See Fourth Communication, page 56.

the complements or merely changed them into inactive derivatives, complementoids.<sup>1</sup>

In order to be certain of the destruction of the zymotoxic group, we heated the sera for fifty minutes to 60° C., a procedure, as shown by numerous subsequent examinations, which absolutely destroys every trace of complement action in the sera so treated.

By treating animals with the sera thus prepared, it is actually very easy to obtain anticomplements. We injected rabbits, guinea-pigs, and dogs with inactive goat serum, and goats and numerous rabbits with inactive horse serum. A parallel series of animals was treated with active serum. The anticomplement action of the serum from the animals treated with complementoids proved fully as strong and often stronger than that of the control animals treated with active serum. By means of the procedure described in detail in our Fourth Communication it was readily shown that these were really *anticomplements*.

The injection of the heated serum, therefore, possesses the same value as that of the unchanged serum.<sup>2</sup> Since, however, according to our view it is the haptophore group which causes the immunity reaction, it follows that *inactivation of the complement has destroyed only the zymotoxic group, leaving the haptophore group intact.*

The important question now arises as to how the presence of complementoids influences the activation of the immune body; for whenever a serum is inactivated by heating a formation of complementoid ensues, and it is well known that such a mixture of immune body and complement is reactivated without any trouble by the addition of complement. It seems therefore as though the presence of the complementoid does not hinder the union of immune body and complement.

On this point we have made special experiments by alternately

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<sup>1</sup> At about the same time, exactly similar considerations led Paul Müller (*Centralblatt f. Bacteriologie*, Vol. 29, No. 5) to attempt the production of anticomplement by the injection of serum which had been heated. In his case, however (immunization with chicken blood), *anti-interbody* was principally developed, while anticomplement could not positively be demonstrated. It is possible that this negative result indicates that not all the complements of the different animal species are able to undergo this metamorphosis into complementoid.

<sup>2</sup> We should like to mention that in addition to this, in the case of the goat treated with inactive horse serum, we observed the development of a powerful coagulin.

inactivating and adding complement without finding that the constantly increasing amount of complementoid hindered the action of the complement. This phenomenon can be explained only by assuming that *in the change to complementoid, the haptophore group of the complement suffers a diminution of its affinity for the complementophile group of the immune body.*

In the toxoids of diphtheria poisons the circumstances are somewhat different, for Ehrlich found that in the hemitoxin zone of the poison spectrum the affinity suffers no change through the formation of toxoid. On the other hand, M. Neisser and Wechsberg in another case, namely that of staphylotoxin, have been able to demonstrate a decrease in affinity occurring with the change into toxoid. This behavior is analogous to that of the complementoids observed by us. Hence no general rules governing the affinities in toxoid and complementoid formation can be laid down; the circumstances must be investigated separately in each case. From what slight differences in the constitution of the molecule enormous differences in affinity may arise is seen by studying certain organic acids. Thus, for example,  $\alpha$  and  $\beta$  resorcylic acids differ from each other merely in the position of the two hydroxyl groups; the constants of their affinities, however, differ from each other by over a hundred times. We may therefore perhaps assume that in our special case it depends on the relative positions of the haptophore and hoxophore group and the corresponding relations thereby determined whether any change in *one* group can retroactively affect the *other*.

### III. Concerning Autoanticomplements.

In the third communication, on isolysins, we pointed out that the organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements and so give rise to autotoxins. Further investigations made by us have confirmed this view, so that one might be justified in speaking of a "*horror autotoxicus*" of the organism. These contrivances are naturally of the highest importance for the existence of the individual. During the individual's life, even under physiological though especially under pathological conditions, the absorption of all material of its own body can and must occur very frequently. The formation of tissue autotoxins would therefore constitute a danger threatening the organism more frequently and much more severely than all

exogenous injuries. We believe that the study of these regulating contrivances is of the greatest importance and according to our present investigations either the disappearance of receptors or the presence of autoantitoxins is foremost among these contrivances. It will therefore be necessary to subject all the factors which are of importance in this respect to a thorough analysis.<sup>1</sup>

We shall now mention a few observations relating to the complements which seem to point to a regulatory contrivance as yet undescribed.

Normal rabbit serum possesses a number of properties which are to be ascribed to the presence of complements. First to be mentioned is the property by means of which freshly derived rabbit serum is able to dissolve guinea-pig blood-cells. This is due to the combined action of a complement and an immune body which is present in the serum in comparatively small amounts. Furthermore, rabbit serum is regularly able to activate an immune body derived by treating rabbits with ox blood.

Now we noticed that rabbits which a week previously had been treated with goat serum (whether active or inactive is immaterial) had completely or almost completely lost these properties, and that these changes persisted for weeks after the injection. Hence it follows that owing to the injection of goat serum, complement normally present had been made to disappear. It was therefore essential that the cause of this remarkable phenomenon be determined. We could next show that frequently the serum of these rabbits in its native state, though more surely after heating to 56° C., is able to prevent the above-described complementary action of *normal* rabbit serum. Hence in the above case normal complement has evidently disappeared from the rabbit treated as described, and has been replaced by an *anticomplement* which we shall have to term an *autoanticomplement*.<sup>2</sup>

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<sup>1</sup> Metalnikoff's interesting observation is only apparently a contradiction of these regulating phenomena. He found that a typical *autospermotoxin* is developed in the blood of guinea-pigs which have been treated with guinea-pig spermatozoa, and that this is able *in vitro* to kill the spermatozoa of the animal itself. But such an injurious action on the spermatozoa does not take place, even in the slightest degree, in the living animal, because, as Metalnikoff's researches show, only the immune body combines with the spermatozoa, not the complement. In this case, therefore, an autotoxin within our meaning, *one that destroys the cells of its own body*, does not exist.

<sup>2</sup> According to the investigations of Dr. M. Neisser and Dr. Wechsberg still

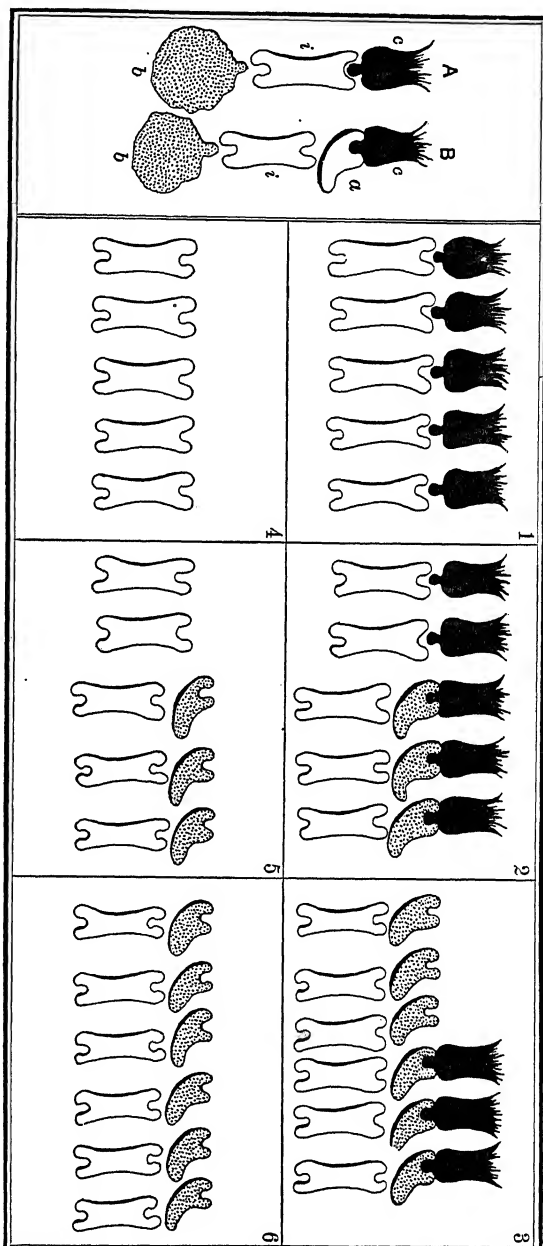
It has previously been shown that such a rabbit serum is rich in antioat complement. We observed an analogous phenomenon, whose nature may perhaps be identical with the above, in a rabbit which had been treated with ox blood (blood-cells and serum) in order to produce a specific hæmolysin. Ten days after the injection of ox blood, the serum failed to show any solvent action whatever on ox blood, in direct contrast to numerous previous cases. At first we thought it possible that no immune body had been formed in this case, for even the addition of an excess of complement in the form of rabbit serum produced no solution. However, on centrifuging the ox blood-cells after treatment with this abnormal serum, and mixing them with salt solution and complement, we found that even slight doses of immune serum caused marked solution. The serum therefore contained plenty of immune body, and this had been anchored by the blood-cells. The presence of this immune body was obscured not only because the complement was absent, but because this had been replaced by an anticomplement which neutralized the complement subsequently added. Because of the anticomplement which it contained, this rabbit serum manifested a marked inhibitory action on the strongly hæmolytic serum of another rabbit (one which had been treated with ox blood).

But what happened in this case after injection of ox blood rarely occurs in such a conspicuous manner. More frequently it is found that the serum in its active state possesses an exceedingly slight solvent action, corresponding to a very small content of complement, and that after heating it manifests a distinct anticomplementary action. This evidently leads to the extreme case above described, as is readily seen when the relations are expressed by means of a diagram. (See figure.)

In studying the question as to how these autoanticomplements are formed, we must constantly bear in mind that normal serum always contains complements in excess. Now it is difficult to see what purpose would be served if at any time the normal complements, so important in cell economy, were paralyzed by autoanticomplements. We shall therefore have to assume that the normal

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in progress, this serum also lacks the power to activate certain *bactericidal* immune bodies. The animals at the same time seem to suffer a decrease in their resisting power against certain infections, a fact which may perhaps serve to exhibit in the purest form the function of certain complements.



A, diagram of the hemolysin; B, action of the anticomplement on the hemolysin; b, blood-cell; i, immune body; c, complement; a, anticomplement. (In this diagram the complementoids are disregarded, since they are here without influence.)

Diagrams 1-6 illustrate the different types developed on treating rabbits with ox blood; 1-3 in the native state, 4-6 in the inactive state.

1 and 4, the usual form. Previous to heating to 56° C., (1), presence of immune body and complement. After heating, (4), free immune body, which can be reactivated by the addition of complement.

2 and 5, abnormal form. Presence of immune body, anticomplement and of complement in slight excess. Corresponding to the partial defection of the complement by the anticomplement, slight haemolytic action of the native serum (2). After heating, immune body and anticomplement are free (5).

3 and 6, abnormal form. Presence of immune body, complement and excess of anticomplement. Corresponding to this, primarily no haemolysis (3), but instead a strong anticomplement action; immune body therefore obscured and only determined by means of the combining experiment described in the text. After heating (6), the immune body and anticomplement are free.



complements circulating in the serum do not cause the formation of autoanticomplements. Confirmation of this view is furnished by the fact that even in animal species possessing identical complements it is impossible to produce anticomplements by means of serum injections. Thus, neither sheep when injected with goat serum, nor, conversely, goats when injected with sheep serum produce any anticomplement, for these two species manifest an extensive similarity in their complements as well as in other serum constituents.

When, then, in spite of this rule, we find that in our case auto-anticomplements have developed, only one explanation remains: that one or the other complement present in the goat serum, *although related, is not identical* with the complement of the rabbit. If we assume that a certain goat complement possesses the *same haptophore group* as does a certain rabbit complement, but that it differs in *the rest of its constitution*, then the assumption that identical complements do not form anticomplements will not apply. In this case, by means of the haptophore group of the particular receptor of the rabbit cell, a *foreign complex* would be anchored which exerts a sufficient stimulus on the cell to cause an increased production and thrusting off of the corresponding side-chains which can functionate as anticomplements.

We shall have to assume that the particular goat complement, because of its identical haptophore group, can be anchored at the same places as the idiocomplements with the same haptophore group. Foremost among these places we may consider the complex receptors which possess two haptophore groups (amboceptors). In this case, contrary to what we usually observe, *the thrusting-off of an amboceptor would be effected through the anchoring of its complementophile group*, and we should then have additional proof for our view that the complex receptors possess two binding groups.

In any case it would seem to be of the greatest importance to gain an insight into the conditions governing the disappearance of the idiocomplements. That they can be caused to disappear through injection of anticomplements produced by immunization follows as a matter of course from our definition of anticomplements. This, however, occurs only under artificial experimental conditions and so possesses but little significance pathologically. Of considerable importance for these occurrences under natural circumstances are the vital conditions governing the disappearance of complement through internal metabolic processes. The origin of the autoanticomplements as it has just been presented by us surely belongs here,



and it has perhaps some practical significance, viz., that in the frequent injection of various curative sera into man and animals, the possibility of autoanticomplement formation should be borne in mind. Another case belonging here has previously been described by us—the disappearance of part of the complements in a rabbit poisoned with phosphorus. In connection with this the following observation of Metalnikoff (l. c.) is of interest. He was immunizing a rabbit with spermatozoa and noticed that in consequences of a purulent process which developed during the course of the immunization, the complement which activated the spermatotoxin disappeared from the serum and did not reappear for a considerable time.

These isolated observations seem to indicate that the complements can disappear during pathological conditions in consequence, perhaps, of a more rapid destruction or of a slower formation. The same holds true for the immune bodies (amboceptors) which in bacteriolysis as well as in hemolysis have at least as great a significance as the complements. Which of these two factors prevails in any single case cannot be decided by any general rule, but each case must be examined separately. Only through such investigation will we gain an insight into the nature of "natural predisposition" and its changes, "increased resistance," "loss of resistance," etc.

## VIII. STUDIES ON HEMOLYSINS<sup>1</sup>

### SIXTH COMMUNICATION

By Prof. Dr. P. LUDWIG and Dr. J. MÖNNINGHOFF

The steady progress of the investigations in immunity is rendered extremely difficult by the fact that in the immunization with living cells and in the study of the immune sera thus obtained a large number of different substances which exist simultaneously is concerned. In our second communication we pointed out that the haemolysins present in *normal* serum, which act on *different* species of blood, are not a single substance in the sense of Buchner's album, and in our fourth communication we showed that this could be demonstrated experimentally by means of elective absorption. It is possible that just as many interferences come into action as the varieties of blood affected. We have also been unable to accept Bordet's unitarian view of the complements. On the contrary, as a result of our own experiments we have become convinced that a large number of complements exist together in blood serum. In like manner Bordet's absorption experiments indicate a plurality of the bacterial agglutinins and those of Mallott a plurality of the normal haemagglutinins. The results of these experiments have been gathered together by M. Neisser<sup>2</sup> in a study in which, on the basis of the same principles, he demonstrates the variety of the *antitoxic* antibodies occurring in normal serum. In conformity to this, the reactive antibodies produced by injections of serum of foreign species are most varied in their nature, and we are only just beginning to gain an insight into their constitution. Aside from the numerous coagulins and antiferments thus produced, it is of the utmost importance, so far as the discussion of immunity problems is concerned, to recognize the fact that the complements

<sup>1</sup> Reprinted from the *Berliner klin. Wochenschr.*, 1901, Nos. 21 and 22.

<sup>2</sup> *Deutsche med. Wochenschr.*, 1900, No. 49.

formed through immunization, corresponding to the multiplicity of the complements present in the serum, are exceedingly manifold.

Especially significant, however, is the fact that the *cells* possess a great number of *different kinds* of groups, which groups can lead to the production of numerous different amboceptors (immune bodies).<sup>1</sup>

Hence in immunizing an animal with cell material, the organism is injected, not with a single uniform substance, but with a multitude of the most varied receptors, each of which is more or less able to produce an antibody. In our fourth communication we defined our point of view on this basis as follows:

"In view of our experiments on isolysins described in our third communication the occurrence of different immune bodies in a hæmolytic serum obtained by immunizing with red blood-cells is not at all surprising. We have obtained a whole series of different isolysins by injecting goats with goat-blood. At present they number twelve. In the red blood-cells not merely a single group, but a large number of different groups, must be considered, which, provided there are fitting receptors, can produce a corresponding series of immune bodies. All of these immune bodies, again, will be anchored by the blood-cells employed in immunization. We may assume that when an animal, species A, is immunized with blood-cells of species B, a hæmolytic serum will be produced which contains a great host of immune bodies. The immune bodies in their entirety are anchored by the blood-cells of species A."

Durham<sup>2</sup> has adopted the same view for the bacterioagglutinins. He assumes a number of "components" (corresponding to our receptors) in the body substance of the bacteria, which can cause the production of a corresponding number of agglutinins. In this way each agglutinin which acts on a certain species of bacteria represents the sum of different kinds of single agglutinins, a view entirely analogous to our assumption of a plurality of immune bodies. This view permits Durham to offer a sufficient and natural explanation of the varying degree of action of typhoid agglutinins on typhoid bacilli of different origin, and of the extension of the agglutinating action of specific sera to related species of bacteria. It would be

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<sup>1</sup> Compare the thorough exposition by Ehrlich in Vol. VIII of Nothnagel's: *Specielle Pathologie und Therapie*, Hölder, Vienna, 1901.

<sup>2</sup> Durham, *Journ. of Experimental Medicine*, New York, Vol. V, No. 4, 1901.

very interesting to see these as yet purely theoretical suggestions of Durham proved by means of experiments.

The pluralistic standpoint adopted by us creates numerous difficulties for thorough analytical work in this field, but it leads to deeper insight into the complicated problems and may perhaps also prove of value in the practical applications in immunity.

### I. Observations on the Pluralistic Conception of the Cellular Immunity Reaction.

To begin, we shall briefly sketch one of the points of view by the plurimistic conception, which seems to be of some practical value. Let us assume that a cell, e.g., a bacterial cell, possesses twenty different groups; then twenty different antibodies corresponding to these will be possible. Each haptophore group of the bacterial cell will then represent an *isolated* point of attack for one particular immune body. It is certainly most logical to conclude the possibility of successfully combating a certain bacterial infection increases directly with the number of kinds of immune bodies that act on the bacterial cell.<sup>1</sup> The ideal effect would obviously be attained if it were possible to produce a serum so constituted as to contain immune bodies for *all* the groups present in the bacterial cell.

The phenomenon of antibody formation as it proceeds according to the side-chain theory is a very complex one, and is composed of a number of phases (binding, super-regeneration, thrusting) which are partly independent of each other. Hence a variety of circumstances may arise which exert an inhibitory action at certain points.

To begin, the cell may be so severely damaged by the action of certain poisonous substances that the formation of antibodies does not occur at all, or occurs in only a very slight degree; for antibody production, which is a kind of regeneration process, presupposes a certain degree of cell efficiency.<sup>2</sup>

This damaging effect will result especially with *highly toxic*

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<sup>1</sup> It is, in fact, conceivable that the occupation of a single group only produces a certain amount of *injury* to the cell without being able to cause its death. The danger to the life of the bacterial cells would increase in proportion to the number of partial injuries, which again would correspond to the increase in the number of types of receptors. It is possible that the potent bactericidal sera obtained owe their success to a certain plurality of the immune bodies.

<sup>2</sup> Weigert has already called attention to this. See Lubarsch-Osborne, *Ergebnisse der Pathologie*, 1897, page 138.

stances, provided the receptors fitting these are present exclusively in vitally important organs, e.g., the central nervous system. This perhaps explains the circumstance that it is exceedingly difficult to produce an antitoxin in mice and guinea-pigs with unchanged tetanus poison, while this is easily effected when toxoids are used. On the other hand, an immunization of rabbits by means of unchanged tetanus poison is very easy to attain, because in these animals, as is shown by the investigations of Donitz and of Roux, the greater part of the receptors lies outside of the poison-endangered central nervous system.

However, even without any development of illness it is not at all *necessary* that antibodies should be produced in every case in which an anchoring occurs. Metchnikoff, for example, has called attention to the fact that with frogs in whom every trace of illness is avoided by keeping them cool (as we know from Courmont's beautiful observations) it is impossible to produce any tetanus antitoxin. Investigations by Morgenroth have confirmed this result and shown further that even by treatment with toxoids under various conditions it is impossible to produce a trace of immunity. Probably in this particular case these results indicate that the regenerative powers of the frog's tissues are not equal to these extraordinary demands.

Such an explanation for failure of the antibody to develop is, however, much less probable in the case of warm-blooded animals; and as the number of experiments increases, these cases are becoming more frequent. Probably all who have busied themselves with the subject will have found, particularly with the artificially produced cell poisons, that in some cases it is extremely difficult if not impossible to effect the production of *anti-immune bodies*. Thus, Metchnikoff injected a series of guinea-pigs with specific spermatoxin, a substance which certainly finds receptors in the guinea pig's organism. Despite this, he found but two cases in which even a suggestion of antispermotoxin could be demonstrated. In the case of a dog injected with a specific dog blood immune body derived from a sheep, we have failed despite long-continued treatment to produce any anti-immune body. With this series of phenomena must also be classed the fact that it is extremely difficult if not impossible in a number of animal species to produce antienzymes by the continued injection of certain enzymes.

The explanation of these facts presents *two* possibilities: First, the receptors concerned in the particular case may be of peculiar

constitution in one respect, i.e. in being firmly bound to the protoplasm, so that a thrusting-off, which is essential for the formation of antibodies, does not occur even with an increased regeneration (*sessile receptors*). This leads to the conception that the regeneration of the receptors may take two courses: (a) a thrusting-off of the receptors ensues, and with this a formation of antibody, (b) in the case of sessile receptors, a hypertrophic process sets in comparable perhaps to a simple muscle hypertrophy, according to Weigert's conception. Second, it is conceivable, as Maggessoth<sup>1</sup> has shown in the immunization against tetanus, that normal preformed regulating contrivances come into action, for, in the case of enzymes (in contrast to toxins we are dealing with substances normally produced by the organism itself). Hence it is possible that the formation of an antienzyme is followed by the production of the enzyme itself, in consequence of an internal regulating contrivance.

In any event these observations will show how the factors just discussed can make it possible, when cells possessing numerous different receptors are injected, that only a small number of the antibodies theoretically possible is actually produced. It is therefore very likely that the immunization of one animal species with a certain kind of cell or bacterium results in the production of only part of the possible antibodies. When, however, the same kind of cell or bacterium is injected into a second animal species, it is highly probable that in this species the haptophore groups of the cells will find a receptor apparatus which in part at least is different from that of the first species. The prerequisite for such a difference is the obvious assumption that the receptor apparatus of one species is not identical with the receptor apparatus of a second not very closely related species. For example, it is possible that a certain haptophore group *a* of the typhoid bacillus finds fitting receptors in the organism of the rabbit, but not in that of the dog, whereas another group *b*, finds just the reverse conditions. If these presumptions are correct an important principle for the practical production of curative sera will follow, namely, that in any single case one would immunize a number of different animal species, select the sera containing different immune bodies, and by mixing these, produce a curative serum containing different types of receptors in as complete a form as possible.

Owing to the importance of this subject we have first under-

<sup>1</sup> *Centralblatt für Bakteriologie*, Vol. 20, 1909.

taken the experimental study of the preliminary question whether immune sera derived by treating two different animal species with the same cells are identical so far as their antibodies are concerned, or whether they are partly or wholly different. Of these antibodies the most important are the bacteriolytic and hæmolytic immune bodies. According to our conception, as is well known, these possess two haptophore groups, one, the *complementophile* group, and the other (which anchors itself to the receptors of the cells causing the immunity) which we can briefly designate the *cytophile* group. According to what has been said above it is this second group which possesses special significance in the question under discussion, and we may therefore formulate our problem as follows: *To determine whether, in the immunization of different animal species with cells of one kind, amboceptors (immune bodies) possessing different cytophile groups arise.*

The experimental study of this question can be pursued in the main in two different ways: 1, by means of the absorption test which, although it is very difficult, is applicable to bacteriolysins as well as to hæmolsins; 2, by neutralization with antiamboceptors (anti-immune bodies).

The latter way, the more elegant of the two, is, however, presumably applicable only to those immune bodies which are directed against cells of the organism. A *hæmolytic* or *cytotoxic* immune body, as is to be expected, always finds points of attack in the organism of the corresponding animal species, for this is the first prerequisite for the possibility of an anti-immune body. As a matter of fact also, such anti-immune bodies have already been observed. On the other hand, the immune bodies of *bactericidal* sera, since their natural counter groups are found in the bacterial cells, will in all probability not find these groups in the cells of the higher animals. Hence it seems improbable, unless by chance they occur in an isolated case, that anti-immune bodies directed against the *bactericidal* immune bodies will be produced.

## II. Concerning the Variety of the Cytophile Groups of Homologous Immune Bodies.

We selected immunization with ox blood-cells as being especially adapted for these experiments. Such immunization had already been carried out by von Dungern on rabbits. The production of immune bodies in high concentration succeeds particularly well in

this case, so that later investigators (Buchner, Rehn, Bulloch) have also employed this useful combination. In many cases, most easily by means of intraperitoneal injections of the ox blood, a potent hæmolsin is produced of which 0.005-0.0005 cc. suffices to dissolve 1 cc. of the 5% ox-blood mixture. Since the production of the immune body is unaccompanied by any increase in complement (as von Dungern showed in just this case) it is always necessary, in order to bring the total amount of immune body into action, to add extra complement. This is found in large amounts in the serum of rabbits and especially in that of guinea-pigs.

Now we have observed that the serum of these rabbits which had been immunized with ox blood is able to dissolve not only the blood-cells of *oxen*, but also those of *goats*. The following table shows a comparison of the solvent action of several of these sera on the blood-cells of oxen and of goats. Guinea-pig serum (0.1 or 0.15 cc.) was used as complement since rabbit serum itself, in the doses required, often exerted a hæmolytic action on the goat blood-cells.

TABLE I.  
ACTION OF THE IMMUNE BODY OF THE RABBIT IMMUNIZED WITH OX BLOOD, ON OX BLOOD, AND ON GOAT BLOOD.

Number of the Rabbit Treated with Ox Blood.	Complete Solvent Dose for 1 cc. of Ox Blood. cc.	Complete Solvent Dose for 1 cc. of Goat Blood. cc.	Ratio of the Solvent Doses (Approximate). Complete Solvent Dose for Ox Blood = 1.
No. 1 of I-24-01.....	0.0042	0.0061	1:1.5
" 2 " XII-14-00.....	0.0035	0.0061	1:1.7
" 3 " II- 8-01.....	0.002	0.0035	1:1.8
" 4 " II- 8-01.....	0.003	0.01	1:3.3
" 5 " I-21-01.....	0.0017	0.0061	1:3.6
" 6 " XII-17-00.....	0.0014	0.0051	1:3.6
" 7 " XII-14-00.....	0.00088	0.0042	1:5
" 8 " II- 3-01.....	0.0051	0.05	1:9.8
" 9 " XII-15-00.....	0.00073	0.0073	1:10
" 10 " II- 9-01.....	0.0035	0.06	1:17

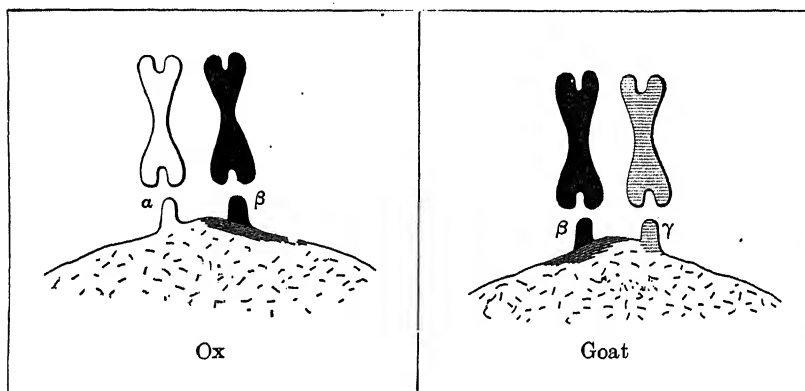
This table shows that the hæmolytic action of the immune body is always less on goat blood than on ox blood, and that the ratio solvent doses for the two species of blood is *not constant* but within fairly wide limits, as can be seen from the last column.



This variable ratio indicates that the solvent action on the two species of blood-cells is not the simple function of one and the same immune body, but that two fractions of immune bodies are present in the serum, of which one acts exclusively on ox blood-cells, while the other fraction acts both on ox blood and on goat blood-cells.

These relations can be studied directly by means of elective absorption. If the immune body is treated with a sufficient amount of ox blood cells and the fluid is then separated by centrifuge, it will be found that the serum has lost its solvent action for *both* species

FIG. 1.



Blood-cell of an ox and of a goat, showing specific and common receptors

of blood; for by means of the ox blood-cells, which as the original excitants of the immunity are carriers of all the receptors in question, both fractions of immune body have been bound. When the same experiment is performed with goat blood-cells, it can be shown that the fluid *has lost its solvent power for goat blood, while that for ox blood remains*. In favorable cases the solvent power for ox blood may remain almost unchanged. The conditions present can be readily understood by reference to Fig. 1.

Let this represent schematically three portions of the combining groups of the blood-cells, of which the first,  $\alpha$ , is present only in

the ox blood-cells, the second,  $\gamma$ , only in goat blood-cells, and the third,  $\beta$ , in both. If a rabbit is injected with ox blood, the antibodies (immune bodies) corresponding to groups  $\alpha$  and  $\beta$  will be formed. Ox blood-cells, by means of their  $\alpha$  and  $\beta$  groups, will then be able to anchor *all* the immune bodies, whereas goat blood-cells will anchor only the immune body of portion  $\beta$ , leaving the immune body of portion  $\alpha$  in the solution.

If, as this explanation assumes, the goat blood-cells possess a certain portion of receptors which are common to goat and ox blood-cells, it is essential that by treating rabbits with *goat* blood an immune body should be obtained which likewise would act on *both* species of blood. This, in fact, is the case. And here, as in the first case, the solvent power for the two species of blood-cells usually differs, though of course the relations are reversed from those in that case, as can be seen by reference to Table II.

TABLE II.

ACTION OF THE IMMUNE BODY OF THE RABBIT WHICH HAD BEEN TREATED WITH GOAT BLOOD, ON GOAT BLOOD, AND OX BLOOD.  
(Reactivation with guinea-pig serum.)

Number of the Rabbit Treated with Goat Blood.	Complete Solvent Dose for 1 cc. of Goat Blood.	Complete Solvent Dose for 1 cc. of Ox Blood.	Ratio of the Solvent Powers (Approximate) Complete Solvent Dose for Goat Blood = 1
	cc.	cc.	
No. 1 of II-28-01. ....	0.01	0.024	1.24
" 2 " I-14-01. ....	0.0061	0.025	1.4
" 3 " II-7-01 <sup>1</sup> . ....	0.0012	0.025	1.20
" 4 " XII-18-00. ....	0.0071	0.25 (almost complete)	1.23

<sup>1</sup> On employing the same serum on a *different* ox blood, 0.05 cc. produced no solution at all, and 0.1 cc. merely a trace. This is evidently due to a casual, individual lack of receptors in the ox blood-cells in question, such as showed itself so frequently in goat blood when we studied isolysins.

Because of these ratios we shall have to assume that the goat blood-cells in this case possess a second system of binding groups which is peculiar to them and represented in the above diagram by  $\gamma$ . They possess these, of course, in addition to the receptors,  $\beta$ , which they have in common with the ox blood-cells. In accordance with this, in the elective absorption test in this case, the goat blood-cells will bind the entire lot of immune bodies; whereas when ox

blood-cells are used, the group  $\gamma$  will be left behind, for this possesses affinity only for the goat blood-cells.

The following protocol shows the results of two series of experiments, which exhibits the effect of such reciprocal binding:

To each 5 cc. of a 5% mixture of ox blood-cells or goat blood-cells (freed from serum by centrifuge) varying amounts of the immune body of a rabbit which had been immunized with ox blood are added. The amount of immune body is seen in the first column of the table; in the second and third columns the complete solvent doses (for ox blood and for goat blood) contained in each specimen are given, as they were determined by tests made at the same time. The mixtures are made up to 6 cc. with physiological salt solution, kept at 37° C. for 1½ hours and then centrifuged. Two equal portions of each of the decanted fluids are then taken and again mixed with corresponding amounts of blood-cells. Finally guinea-pig serum is added to activate the mixtures. The hemolytic action which the decanted portions exerted on ox blood-cells and on goat blood-cells is seen in the table. (See Table III.)

The union of the immune body with the *ox blood-cells* has resulted in a considerable abstraction of *both* portions of immune body. On the other hand, the union with *goat blood cells*, by which the action of the fluid is considerably decreased for *goat blood cells*, causes very little decrease in the solvent power for ox blood.

In contrast to this experiment we here reproduce an analogous experiment which shows a directly opposite behavior of the two fractions of immune body of a rabbit immunized with *goat blood*. (See Table IV.)

*Here the goat blood-cells bind both portions of the immune body, while after treatment with ox blood-cells the fraction acting on goat blood is left almost intact.*

Hence by means of this *crossed immunization and reciprocal elective absorption* we succeed in demonstrating that in the case of the rabbits treated respectively with goat blood and ox blood two large fractions of immune bodies can be separated. Of these, one fraction is common to both sera; the other is peculiar to each of them. The main groups of receptors shown in the above illustration and designated  $\alpha$  and  $\beta$  for ox blood, and  $\beta$  and  $\gamma$  for goat blood, are thus to be differentiated.

We have deemed it important to supplement this analysis by experiments on a second species of animal, and have therefore treated

a goat with ox blood. Naturally the serum of the goat so treated dissolves ox blood-cells. Besides this, however, it manifests the ability to dissolve the blood-cells of a few other goats, and therefore contains true isolysins such as we have previously produced by treating goats with goat blood. Thus 0.025 cc. of the serum of

TABLE III.

BINDING OF THE IMMUNE BODY OF A RABBIT TREATED WITH OX BLOOD, WITH OX BLOOD-CELLS, AND GOAT BLOOD-CELLS

Amount of the Immune Body Added. (Derived from a Rabbit by Treating with Ox Blood.)		Number of Solvent Doses Contained Therein.		Solvent Power of the Decanted Fluids.			
				A, after Binding with Ox Blood.		B, after Binding with Goat Blood.	
				(a) On Ox Blood.	(b) On Goat Blood.	(a) On Ox Blood.	(b) On Goat Blood.
No.	cc.	(a) For Ox Blood.	(b) For Goat Blood.				
1	0.001	$\frac{1}{8}$	$\frac{1}{256}$	0	0	0	0
2	0.002	$\frac{1}{4}$	$\frac{1}{64}$	0	0	trace	0
3	0.003	$\frac{1}{2}$	$\frac{1}{32}$	0	0	very little	0
4	0.004	$\frac{3}{4}$	$\frac{1}{8}$	0	0	very little to little	0
5	0.005	$\frac{5}{8}$	$\frac{1}{4}$	0	0	mod to little	0
6	0.006	1	$\frac{1}{16}$	0	0	moderate	0
7	0.007	$1\frac{1}{8}$	$\frac{1}{8}$	0	0	"	0
8	0.008	$1\frac{1}{4}$	$\frac{1}{4}$	0	0	alm't comp	0
9	0.01	$1\frac{3}{4}$	$\frac{3}{8}$	0	0	complete	0
10	0.012	2	$\frac{1}{2}$	0	0	"	faint trace
11	0.016	$2\frac{1}{2}$	$\frac{3}{4}$	0	0	"	faint trace
12	0.02	$3\frac{1}{4}$	1	faint trace	very little	"	very little, top
13	0.024	4	$1\frac{1}{2}$	very little	"	"	little, top
14	0.032	$5\frac{1}{4}$	$1\frac{3}{4}$	lit. to mod.	little to very little	"	little
15	0.048	8	$2\frac{1}{2}$	"	little	"	"
16	0.06	10	3	alm't comp	moderate	"	"
17	0.08	$13\frac{1}{4}$	4	complete	fair	"	"
18	0.1	$16\frac{1}{2}$	5	complete	strong to alm't comp.	"	little to mod.
19	0.14	$23\frac{1}{2}$	7	"	complete	"	mod to little

one of our goats, on the addition of complement, dissolved the usual amount of ox blood-cells. This serum, however, dissolved but two out of five different specimens of goat blood, and the isolysin constituent was present in only very small amounts, so that it required 0.75 cc. serum (thirty times the above amount) to effect complete hæmolysis of sensitive goat blood-cells. Hence in this case also the development of all such amboceptors as could find a point of

attachment (receptor) in the blood-cells of the individual<sup>1</sup> goat itself has been avoided, and the phenomenon which we have previously designated as a "horror autotoxicus"<sup>1</sup> is again presented.

TABLE IV.

BINDING OF THE IMMUNE BODY OF A RABBIT IMMUNIZED WITH GOAT BLOOD, ON OX BLOOD AND GOAT BLOOD-CELLS.

Amount of the Immune Body Added, (Derived from a Rabbit by Treating with Goat Blood)		Number of Solvent Doses Contained Therein.		Solvent Power of the Decanted Fluids.			
				A. After Binding with Ox Blood.		B. After Binding with Goat Blood.	
				(a) For Ox Blood.	(b) For Goat Blood.	(a) For Ox Blood.	(b) For Goat Blood.
No.	cc.						
1	0.038	1	1	0	fair to moderate	0	0
2	0.05	1	1	0	almost complete	0	0
3	0.062	1	1 1/2	0	complete	0	0
4	0.074	1	2	0	"	0	0
5	0.1	1	2 1/2	0	"	0	minimal trace
6	0.13	1	3 1/2	0	"	0	trace
7	0.15	1	4	0	"	0	"
8	0.2	2	5	0	"	0	very little
9	0.25	2	6 1/2	0	"	0	little
10	0.3	3	8	0	"	0	"
11	0.38	3	10	0	"	0	fair to strong

From this experiment we can at once conclude that this receptor system  $\beta$  actually consists of different components, of which only those separate amboceptors (immune bodies) are found in the serum of goats treated with ox blood whose receptors are absent in the blood-cells of the goat itself.

The most important result of these investigations—investigations complete in themselves—is this: *By treating animals with ox blood, two fractions of immune bodies are formed, of which one acts only on ox blood and the other also on goat blood; whereas by treatment with goat blood the contrary though entirely analogous result ensues. These two frac-*

<sup>1</sup> We were also able to observe that the immune body of the rabbits which had been immunized with ox blood and goat blood acted also on sheep blood. Closer investigation would probably show that this behavior is analogous to its action on goat blood. This corresponds entirely to our earlier observations on the extensive similarity of the receptor apparatus of goat and sheep blood as it was manifested particularly by the experiments on isolysis.

tions do not correspond to two different single immune bodies, but each fraction includes several, perhaps an entire host of immune bodies.

The experiments also lead to conclusions of considerable importance in another direction, namely, as affecting our conception of cellular specificity and of the specificity of reaction products. Heretofore it has been held that the injection of blood of species *a* results in a specific immune serum, i.e. one acting only on *a*; and even Metchnikoff<sup>1</sup> has recently expressed this view. We had already become acquainted with certain exceptions to this principle. The isolysin, for example, produced by injecting goats with goat blood, also dissolves sheep blood; and, *vice versa*, the immune body of goats which have been injected with sheep blood acts also as an isolysin. At that time we emphasized that these results are only to be explained by assuming that certain types of receptors are common to both species of blood. The same holds true in the case under discussion. von Dungern<sup>2</sup> has come to the same conclusion as a result of his experiments. He found that the immune body produced by injection of ciliated epithelium acts also on the blood-cells of the same species, and that conversely the hemolytic immune body produced by injection of blood-cells is partially bound by ciliated epithelium.

All these circumstances indicate that we must not regard the specificity of the immune bodies from the conception of specificity employed in systematic zoology and botany. The immune sera, as we have often mentioned, are not of simple unitarian nature, but consist of a series of single immune bodies whose cytophile haptophore groups correspond to the receptors of the exciting cells. Hence such an immune serum will be able to affect all such elements which possess any one of the receptors whose type is common to those elements and the original cell "*a*." The influence exerted by the immune serum will be powerful in proportion to the extent of this correspondence of receptors. Now we have reason to believe (cf. Ehrlich's deductions, l. c., and Weigert's in Lubarsch-Ostertag's *Ergebnisse der Pathologie*, 1887, p. 141) that certain receptors are very widely distributed among various animal species. Thus the blood-cells of a large number of species possess receptors fitting ricin, abrin, croton, and tetanolysin, and ganglion cells of the most divergent animals possess receptors for tetano-

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<sup>1</sup> Revue générale des sciences, 1901, No. 1.

<sup>2</sup> See page 47.

*spasmin* or for *sausage poison*. Within the animal organism, in like manner, certain receptors are evidently widely distributed in the most varied organs, as is shown, for example, by the experiments with tetanus poison. Looked at from this standpoint, the apparent deviations in specificity are comprehensible. We are convinced that in this field the near future will furnish us with extensive material of immense value in the analysis and study of the distribution of receptors. We are led to conclude, therefore, that in the production of immune bodies by immunizing with cells we can speak of *specificity* only in the sense that there is always a specific relation between the separate types of immune bodies and the receptors.

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The foregoing experiments constitute conclusive proof of the plurality of the immune bodies produced by injections of ox blood and goat blood. We next endeavored to extend these results by effecting a differentiation of various groups of immune bodies by means of the *anti-immune bodies*.<sup>1</sup> The highest concentration of immune bodies at our disposal was the serum of a rabbit which had been immunized with ox blood. For various reasons we chose goats for these immunizing experiments, for we knew that their blood-cells already contained receptors capable of binding a portion of the mixed immune bodies. In treating these goats we used the inactive serum of a rabbit immunized with ox blood. This serum, which was of the highest possible strength, was injected subcutaneously. During the course of two months we had thus injected 120 cc. of an immune body serum, of which 0.005 cc. sufficed, when reactivated with guinea-pig serum, to completely dissolve 1 cc. of a 5% mixture of ox blood cells. At the end of that time we were able to demonstrate the existence of an anti-immune body of considerable protective power. That this was really an *anti-immune body* which inhibited the anchoring of the immune body to the red blood-cells, is seen by the following combining experiment.

0.5 cc. of the anti-immune body (inactive serum of a goat treated as just described) are mixed with varying amounts of the immune body (inactive serum) of a rabbit treated with ox blood. Thereupon 1 cc. of a 5% mixture of blood-cells is added to each specimen. These are then kept at 40° C. for one hour and centrifuged. The various sediments are then mixed with salt solution and 0.15 cc. normal guinea-pig serum. A parallel experiment (control test) is made in

<sup>1</sup> See Ehrlich's recent study, page 573.



which the anti-immune body is replaced by the same amount (0.5 cc.) of inactive normal goat serum. The degree of solution is shown in Table V.

TABLE V.

Amount of Immune Body Added. cc.		Number of Complete Solvent Doses Therein.	Degree of Solution After Addition of Complement.	Degree of Solution of the Sediment in the Control Test.
No. 1	0.00125	1	no solution	complete solution
" 2	0.0025	2	" "	" "
" 3	0.00375	3	" "	" "
" 4	0.005	4	trace solution	" "
" 5	0.0075	6	little solution	" "
" 6	0.01	8	almost complete solution	" "
" 7	0.025	20	complete solution	" "

From these figures we see that a *single* solvent dose becomes available for combination with the red blood-cells only after eight times the solvent dose has been added, and that a triple dose is completely neutralized, i.e., prevented from combining with the blood-cell. The control test shows that 0.5 cc. of a *normal* inactive goat serum does not prevent the combination of a *single* solvent dose of immune body (0.00125 cc.). The sediment in this case is completely dissolved on the addition of complement.<sup>1</sup> By this experiment the inhibiting substance is definitely characterized as an *anti-immune body*. The following example will show the exact quantitative relation of this anti-immune body.

Each 0.4 cc. inactivated serum (anti-immune body) of the goat treated with immune body are mixed with the given amount of inactive serum (immune body) of a rabbit treated with ox blood. The specimens are made up to the same volume by the addition of salt solution, kept at room temperature for half an hour, and then mixed with 1 cc. of a 5% suspension of ox blood, and with 0.15 cc. normal guinea-pig serum (complement). A control test is made in which normal inactive goat serum is used instead of the anti-immune body. (See Table VI.)

<sup>1</sup>We should like to remark that in the course of numerous experiments we have now and then found normal goat sera containing slight amounts of an anti-immune body acting on the immune body of rabbits treated with ox blood. This is to be brought into connection with the law (see also Neisser, l. c.) that the artificially produced antibodies frequently represent only an increase of normal functions.



TABLE VI.

Experiment with 0.4 cc. Anti-immune Body.		Control Test with 0.4 cc. Normal Goat Serum.	
Amount of Immune Body. cc.	Solvent Action.	Amount of Immune Body. cc.	Solvent Action.
0.0175	complete solution	0.001	complete solution
0.0145	strong "	0.00085	almost complete solution
0.012	fairly strong solution	0.0007	strong solution
0.01	moderate solution	0.0006	" "
0.0085	little solution	0.0005	moderate solution
0.007	" "		
0.006	trace solution		
0.005	small trace solution		
0.0044	" " "		
0.00375	" " "		
0.003	minimal trace solution		
0.0025	" " "		
0.002	" " "		
0.0018	0		

This shows that 0.2 cc. of the anti-immune body are able to completely inhibit the action of 1.8 times the solvent dose of immune body as determined by the control test, and that it almost neutralizes the action of five times such a dose. If, however, we measure the protective power by comparing the complete solvent doses in the two cases, this appears much stronger. The ratio of the complete solvent doses in the presence of immune body and in the control test is then 1:17.5. We shall discuss the reason for this later.

Since the inactive rabbit serum employed in immunization contained complementoids, the presence of anticomplements along with the anti-immune body is easily understood. The anticomplements at first were directed against rabbit serum. After the immunization had continued for some time longer anticomplements appeared directed against certain complements of *guinea-pig serum*. In these experiments, therefore, in order to overcome the anticomplement action (in reality insignificant) directed against the reactivating guinea-pig serum, it was merely necessary to employ a considerable excess of the latter.

In contrast to these results are those obtained in an analogous series of experiments, in which, however, *the complement was in the form of goat serum instead of guinea-pig serum*. (See Table VIa.)

TABLE VIa.

Experiment with 0.4 cc. Anti-immune Body.		Control Test with 0.4 cc. Normal Goat Serum	
Amount of Immune Body, cc.	Solvent Action.	Amount of Immune Body, cc.	Solvent Action.
0.051	complete solution	0.051	complete solution
0.042	almost complete solution	0.042	almost complete solution
0.029	moderate solution	0.029	moderate solution
0.02	trace solution	0.02	very little solution
0.017	faint trace solution	0.017	trace solution
0.014	0	0.014	0

*In this combination the anti-immune body exerts no action. Hence we must here be dealing with a particular type of immune body which effects a combination with a complement present in goat serum. This immune body enters into no relation with the complex of immune bodies here present; it must therefore possess a haptophore group which finds no fitting counter group therein.*

As a matter of fact the completion by means of goat serum occupies a special position, for the quantitative relations of the immune body are entirely different from those observed when guinea-pig serum is used. In order to effect complete solution when goat serum is used as complement, it is necessary, as a rule, to use from ten to thirty times the amount of immune body that would be required if guinea-pig serum were used as complement. This is well shown by Table VII.

TABLE VII.

No.	Complete Solvent Dose of Immune Body when Complemented with Guinea-pig Serum 0.15. cc.	Complete Solvent Dose of Immune Body when Complemented with Goat Serum 0.5. cc.	Ratio of the Two Doses.
1	0.005	0.05	1:10
2	0.0075	0.075	1:10
3	0.0075	0.1	1:13
4	0.0025	0.075	1:30

That this behavior is not due to a smaller content of complement in the goat serum can readily be determined by suitable experiments especially by increasing the dose of the latter.

This can only be explained by assuming that only part of the total number of immune bodies find fitting complements in goat serum, and that this partial number varies, but is always less than the number of immune bodies activated by guinea-pig serum. The diagram presented below will best make this relation clear.

We have made a further series of experiments in order to complete these studies, and have discovered that our anti-immune body also protected *goat blood-cells* against the immune body derived from a rabbit treated with ox blood. This of course is quite natural, for we have already shown that this action on a strange species of blood depends on a concordance of certain haptophore groups. Similarly, the anti-immune body protects *ox blood-cells* against the immune body of a rabbit immunized with *goat blood*.

These experiments lead us to the following conclusions: *The anti-immune body derived by injecting goats with immune bodies of rabbits is not a simple uniform (einheitlich) substance, but is made up of a whole series of partial immune bodies. In the ox blood used to immunize the rabbits we have already distinguished two main portions of receptors to which again two main portions of the resulting immune body correspond. Each of the latter portions in all probability contains a host of partial immune bodies, and we must assume that, corresponding to this, the anti-immune bodies also possess a complex constitution.*

In the following diagram it is not sought to express that the immune bodies which can be activated by guinea-pig serum are all identical. On the contrary each group may represent a different kind of immune body.

We have seen that there is a great difference between the dose of immune body which is *completely neutralized* by a certain amount of anti-immune body, and that which in the presence of anti-immune body causes complete solution. This can be understood when we recall the above-mentioned distribution of partial immune bodies, and examine the diagram, Fig. 2.

In order to choose a simple illustration let us assume that, corresponding to the diagram, the immune serum of the rabbit immunized with ox blood contains only two different types of immune bodies and these, furthermore, in unequal amounts. Let the main portion be represented by immune body type *a*, which is activated by a particular complement present in the animal's own (rabbit's) serum. Further, let the second portion, present in much less amount,

be represented by immune body type *b*, which is activated by a different complement also present in rabbit serum but found in *goat serum* as well. Let the proportion of *a*:*b* be as 10:1; i.e., a quantity of immune serum containing *one* complete solvent dose of *b* will contain *ten* solvent doses of *a*. In this case then it will require ten times as much of the immune serum to effect complete solution by means of immune body *b* (which is the case when goat serum, which contains complement only for *b*, serves for reactivation) used when immune body *a* is employed. The composition of this immune serum can be represented by the formula  $10a + 1b$ .

FIG. 2.

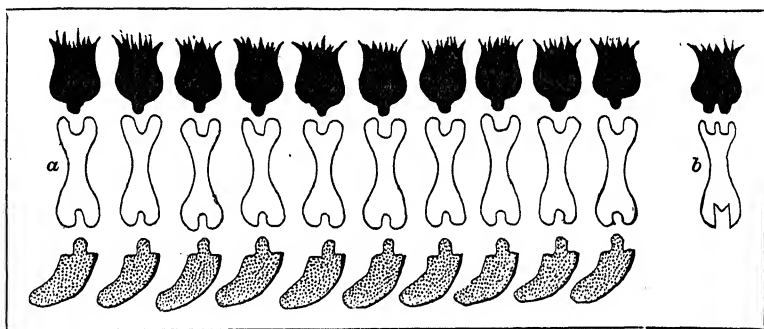


Diagram to show the two types of immune bodies present in the immune serum of a rabbit treated with ox blood. Each immune body symbol corresponds to *one* solvent dose for the amount of ox blood employed in the experiment. Immune body type *a* is present in ten times the amount of type *b*. The complementophile groups of *a* and *b* differ; hence also the complements differ. The anti-immune body serum possesses anti-immune bodies only against *a*.

As is seen by the experiments, an anti-immune body exists only against immune body type *a*. If therefore to an amount of immune body which contains one solvent dose of immune body *b* and ten solvent doses of immune body *a* (i.e.,  $10a + 1b$ ) a *large quantity* of *anti-immune body serum* is added, and then sufficient suitable complement it will be found that solution always occurs, for the reason that a single solvent dose of *b* is present which is unaffected by the anti-immune body although this was able to neutralize ten solvent doses of *a*. *One-tenth* of the above amount of immune body, on the other

hand, will be *completely inhibited* in its action. For this contains *one* complete solvent dose of immune body *a* which is neutralized by the anti-immune body, and only *one-tenth* of a solvent dose of *b* which, although not affected by the anti-immune body, is so slight as not to cause any appreciable solution. Only when larger amounts of immune body are employed in which *b* becomes active does solution occur, and this becomes complete only when that quantity is reached which contains  $10a + 1b$ . Naturally, if the ratio is 1:20, a quantity will be required which can be represented by the formula  $20a + 1b$ .

These explanations will perhaps suffice to make the above-mentioned peculiarities in the action of the anti-immune body comprehensible. They will perhaps also make clear that *between the dose of immune body whose action is completely inhibited by the anti-immune-body serum and the dose which causes complete solution a large number of intermediary stages exist in which the degree of solution gradually increases.*

In reality the circumstances are much more complicated than this; for with the increase in the dose of immune body a large number of new immune bodies, similarly superposed, come into action—immune bodies which find few or no corresponding anti-immune bodies in the antiserum.

This brings us to another important question: *Is it possible by means of the anti-immune body to demonstrate the difference of the immune bodies produced by injections of ox blood into different species?*

Our first experiments were undertaken with the serum of goats which had been immunized with ox blood. As will be seen by the following figures, our anti-immune body (derived by injections of an immune body obtained from rabbits) in this case exerts no action. The varying amounts of immune body mentioned are mixed with 0.4 cc. anti-immune body and then with 1 cc. 5% ox blood suspension and 0.5 cc. normal active goat serum to activate the mixture. In the control test 0.4 cc. inactive normal goat serum are used instead of the anti-immune body. (See Table VIII.)

That this serum differed markedly with respect to its content of individual immune bodies was already shown by the fact that, in contrast to the serum of immunized rabbits, it did not possess a hemolysin acting on all goat blood-cells in general, since such a hemolysin would have exerted a most injurious action in the form of an autolysin. As a matter of fact, the law already mentioned under the name "*horror autotoxicus*" applied here also, and hence merely an *isohemolysin*.

was developed which acted only on goat blood-cells of a *few* individuals and which therefore possessed only a few individual special groups in its immune bodies. Against this isolysin, which represented a relatively small portion of the types of immune bodies found in the goat, our anti-immune body also proved entirely ineffective, as is seen in Table IX.

TABLE VIII.

Experiment with Anti immune Body.		Control Test.	
Amount of Immune Body, cc.	Solvent Action.	Amount of Immune Body, cc.	Solvent Action.
0.051	complete solution	0.051	complete solution
0.042	almost complete solution	0.042	almost completely dissolved
0.035	strong solution	0.035	almost dissolved
0.029	moderate solution	0.029	moderate solution
0.02	trace solution	0.02	very little solution
0.017	doubtful	0.017	trace solution
0.014	0	0.014	0

In this experiment the method was exactly similar to that of the previous ones. The blood was from goat No. III, 1 cc. of a 5% suspension being used.

TABLE IX.

Experiment with 0.4 cc. Anti-immune Body.		Control Test with 0.4 cc. Normal Inactive Goat Serum.	
Amount of Immune Body, cc.	Solvent Action.	Amount of Immune Body, cc.	Solvent Action.
1.5	complete	1.5	complete
1.0	strong	1.0	strong
0.88	strong	0.88	strong
0.61	moderate	0.61	moderate
0.51	little	0.51	little
0.42	trace	0.42	trace
0.35	0	0.35	0

We see from this that by treating a goat with ox blood-cells, immune bodies have been formed the main portion of which differs from those obtained by immunizing rabbits with ox blood or goat blood.

A second species of animal in which we have been able to demonstrate a difference in the immune bodies is the goose. The immune bodies obtained by injecting a goose with ox blood-cells are also not in

the least affected by our anti-immune body. It may be that an entirely different receptor apparatus is present in the goose and that this effects a combination with different haptophore groups which leads to the formation of immune bodies of entirely different character.

Our further experiments concerned themselves with the action exerted by our anti-immune body on immune bodies derived from *rats*, *guinea-pigs*, and *dogs* by treatment with ox blood. We found that the anti-immune body exerted a distinct protective action against all three sera, but that this was less strong than that against the immune body of the rabbit. The protection was least against the serum of the rat, for it did not even suffice to absolutely protect against one-half or one-third of the fatal dose. Complete solution ensued in the presence of 0.3 cc. anti-immune body even when only double the usual solvent dose of immune body was employed. This indicates that this serum contains a relatively large amount of the non-neutralizable types of immune bodies, in any case an amount far greater than is contained in the rabbit serum. In the guinea-pig the case is very similar, the proportion of double the solvent doses being as 1:3. The nearest approach to the ratio as found in the rabbit is seen in the serum of a dog treated with ox blood. In this it required six times the usual solvent dose to effect complete solution in the presence of the anti-immune body.<sup>1</sup>

All this leads to the conclusion that in the immune serum of these three species the cytophile group of certain portions is identical with the cytophile group of certain immune bodies in the rabbit. Certain particular groups of the ox blood-cells therefore must fit equally into the receptors of these different animals. In view of this fact, the entire absence in the goat of that portion of immune body which can be neutralized by the anti-immune body is of special interest. As already stated, we are here dealing with an exception which is connected with the impossibility of autolysin formation.

We must therefore conclude that in conformity with our assumption, the immune bodies formed in any single case by treating various

<sup>1</sup> It is perhaps of interest to know that the immune bodies derived from these three species (guinea pig, rat, and dog) differed in their behavior toward *goat blood-cells*. It was found that while the immune bodies of guinea pigs and rats acted on goat blood, those of the dog did not. This indicates that the dog, in contrast to rabbits, guinea pigs, and rats, possesses no receptors for the groups (1) of the diagram, Fig. 1, common to the blood cells of oxen and goats.



animals with ox blood-cells are not, as a matter of fact, of simple [einheitlich] nature. Those obtained from goats and geese are very markedly, if not entirely, different from those of rabbits, while those from guinea-pigs, rats, and dogs are partly so.

We have already pointed out the significance of this circumstance in § II, page 92. In all probability similar conditions obtain for bacteria, and it would therefore be advisable *not* to attempt the production of bactericidal sera from a single animal species, as is now customary, but to make a preparation containing a mixture of immune sera derived from animals whose receptor apparatus are as divergent as possible.

### III. Concerning the Variety of the Complementophile Groups of Homologous Immune Bodies.<sup>1</sup>

From the foregoing sections it will be seen that in combating infectious diseases we believe it advisable to employ simultaneously a great many bactericidal immune bodies which, in conformity with the multiplicity of groups in the bacterial cell, will differ in their *cytophile* group. It will now be necessary to investigate the question of a difference in the complementophile groups of these immune bodies. However, the treatment of this question can at present only be fragmentary, since our methods in this field are still very incomplete and definite results can only be obtained in specially favorable cases.

It will be advisable to commence this study with the immune serum of a rabbit treated with ox blood. In this it has already been pointed out that two portions of immune bodies are present, each of which again is to be regarded as composed of a number of partial-immune bodies. This view of the composition of the immune bodies is supported by the reactivating experiments in which a number of different kinds of sera furnished the complements. This brings us to our present topic.

We have already mentioned that the most favorable results are achieved when our immune body is activated by rabbit or guinea-pig serum; the activation by means of goat serum, together with its peculiarities, has also been discussed at length.

The following list of complements shows their action in the presence of varying amounts of an immune body from a rabbit immunized with ox blood. The amount of complement employed was always ample.

<sup>1</sup> See also Ehrlich's later views, page 560.



TABLE X.

Activating Serum.	Amount of Immune Body with which Complete Solution Occurs, cc.
Guinea-pig serum	0.0025
Rabbit serum	0.005
Rat serum	0.005
Goose serum	0.015
Chicken serum	0.015
Goat serum	0.05
Pigeon serum	no complement action
Horse serum <sup>1</sup>	" " "

<sup>1</sup> This horse serum, which had been freshly obtained, failed also to reactivate the immune bodies of a goat and a goose which had been immunized with ox blood. Yet it was not at all free from complement, for even in amounts of 0.15 cc. it dissolved guinea-pig blood completely. It did not act on rabbit blood.

This shows that when different sera are used as complements there is a great variation in the amount of immune body necessary for solution. Especially the extreme cases make it seem probable that we are dealing with different types of partial-immune bodies, to which different complements in the serum of the individual species correspond. That the complements of *different species* are not identical is admitted even by Bordet, although he recognizes only a single complement for each species.

That these complements are anchored to the corresponding immune body by means of a haptophore group may practically be regarded as proven, (1) by our experiments with blood-cells which had been laden with immune body, and (2) by the demonstration of anti-complements which diverted the complements from the immune body.

According to our view the point at which the haptophore group takes hold is situated in the complementophile portion of the immune body. Hence we formerly designated the latter as "*interbody*"; recently we term it "*ambceptor*." A number of special investigators have accepted this view, as can be seen from the designations used by them; thus P. Müller, "*copula*"; London, "*desmon*"; Metchnikoff: "*cytase*" = complement; "*phalocytase*" = immune body.

Consequently we arrive at the view that in the mixture of immune bodies in the case under discussion a number of different complements

*trophile groups come into play.* With the means at present at our disposal it is impossible, except in a few favorable cases, to determine whether this plurality of complementophile groups corresponds exactly to a like plurality of cytophile groups. A case in point is that of the partial immune body which is reactivated by goat serum, for which we could show that it was not diverted by our anti-immune body.<sup>1</sup>

The difficulty of a full analysis of these cases is due especially to the many possibilities that must be considered. It is possible that immune bodies with different cytophile groups possess the same complementophile group, or that those with the same cytophile group possess different complementophile groups; and finally it is possible that, besides a particular cytophile group, an immune body may possess two, three, or more complementophile groups (*triceptor*, *quadriceptor*).

In any case it may be considered a fact that in the immune-body mixture different kinds of complementophile groups come into play. Were we to assume that the serum of an animal species contains only a single complement, we should have to regard such a plurality of complementophile groups as evidently a useless arrangement. It seems incredible that a given organism should form haptophore groups in its cells (for the immune bodies are merely thrust-off cell derivatives) if these groups were never during life to come into action, but were only to be of service in case the organism were injected with foreign cells. It is much simpler and more natural to view these circumstances from our standpoint, namely, *that the complements of an animal are, from the first, of manifold variety.*

This assumption best harmonizes the results of the various experiments which we have made from the beginning of our studies in hæmolysis. By filtering goat and horse sera through Pukall filters we were able to demonstrate two complements. One of these, fitting

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<sup>1</sup> In our fourth communication we have discussed analogous cases in detail, subjecting them to thorough experimental investigations. At that time, however, our studies were directed only to the complementophile groups. In that case the serum of guinea-pigs immunized with rabbit blood contained two immune bodies, of which one found its complement in guinea-pig serum but not in rabbit serum. These immune bodies were present in the proportion of 1:10. In another case mentioned at that time we observed considerable chronological variations in the proportion of two immune bodies with different complementophile groups.

to an immune body acting on rabbit blood, passed through with the greatest difficulty; the other, fitting an immune body acting on guinea-pig blood, passed through in part completely isolated. We were further able to show that heating the serum of a buck treated with sheep blood caused all the complements excepting one to disappear. The one which withstood the heat fitted the immune body developed by the immunization. We were able to demonstrate the same *thermostabile* complement in greater or smaller amounts in the serum of normal goats and calves. To again call attention to these experiments is not superfluous, for Gengou (*Annal. l'Inst. Pasteur*, 1901) in spite of these proofs of the plurality of complements, still maintains that the serum of each species contains only a single simple complement, "*the alexin*."

It would be natural to conclude that there is a plurality of complements from the manifold variations observed in the completion of various inactive sera by normal sera. The commonest example of this, probably known to every one having experience in this field, consists in the fact that a certain immune serum can be activated by *two* different sera serving as complement, whereas other immune sera can be activated by only *one* of these sera. Nevertheless from our standpoint we cannot regard this method of proof as at all conclusive because it rests on the assumption that for a certain species of blood a serum contains only a *single* interbody (or immune body). In our fourth communication we have already shown that this assumption does not hold, even for the interbodies of normal sera.

*The assumption of a plurality of complements in normal sera is supported by the fact that by injections of a normal serum (which, according to our view, possesses various active substances which may be present as complements, or, at times, in the form of complementoids) antisera are formed which act against the complements of various other sera. In a number of different animals by injecting various sera we have succeeded in obtaining anticomplements acting not only against the serum originally employed, but also against certain complements of rabbits and guinea-pigs. According to Bordet's experiments it is possible, by injecting a rabbit with guinea-pig serum, to obtain an isolated anticomplement against a complement (able to act in this particular case) present in guinea-pig serum. From this it follows that in these sera, since they excite the production of different anticomplements, at least two different complements*

are concerned. In this connection it is particularly interesting to note that by long-continued treatment of a goat with *rabbit serum* we obtained an *anticomplement serum* which acted also against *guinea-pig serum*. Table XI will make this clear. All of the experiments are made with an immune body derived from a rabbit by immunizing with ox blood.

TABLE XI.

Anticomplement Derived from	Treated with	Protection against Rabbit Complement.	Protection against Guinea-pig Complement.
Rabbit	Guinea-pig serum.....	+	+++
Goat	Dog serum.....	+++	+++
Goat	Horse serum.....	+++	+++
Goat	Rabbit serum.....	+++	+++
Rabbit	Goat serum.....	++	++
Rabbit	Sheep serum.....	+++	+++

+++ = strong protection; ++ = fairly strong protection; + = very slight protection.

With the assumption of a plurality of complements we are led to the view that the various complementophile groups of the immune body here concerned (contained in rabbit serum) are complemented by a like number of partial complements. As a result of this fact the possibility exists that certain of these complements are not constant, occurring in the blood only from time to time.

We may perhaps give another example of these partial complements, which concerns one of a number of rabbits treated with repeated injections of goat serum. As already described in a previous communication, this results in the disappearance of certain complements and their replacement by corresponding autoanticomplements. In the example mentioned, this disappearance manifested itself by the fact that large amounts of the rabbit serum were unable to activate the single or the double fatal dose of the immune body from a rabbit immunized with ox blood. However, when thirty times the amount of immune body was employed complete solution ensued. Evidently the principal portion of the complements usually present had disappeared from this serum, but a partial complement had remained which acted on a partial-immune body present in relatively small amounts. The circumstances in this case therefore are entirely analogous to those above described in

which we proved that a particular immune body present in small amounts and not diverted by our anti-immune body, finds a complement in its own serum which, in contrast to the other complements, is present also in goat serum.

Three things have thus been established:

1. Each normal serum contains a number of different complements;
2. In different animals a part of the complements present are either completely similar or at least similar in their haptophore groups;
3. The immune bodies obtained in an animal species represent a number of different complementophile groups.

As a result of this demonstration the question whether or not the resultant immune-body mixtures obtained in different animals are identical in their complementophile portion loses in interest at least so far as the problems under discussion are concerned.

Hence we should merely like to add to the results obtained by activating the immune body of a rabbit immunized with ox blood, the results of a parallel series of experiments made at that time with the same amounts of reactivating sera *but with the immune body from a goose immunized with ox blood.* (See Table XII.)

TABLE XII.

Reactivating Normal Sera.	Amount of the Rabbit Immune Body Sufficient to Effect Complete Solution. cc.	Amount of the Goose Immune Body Sufficient to Effect Complete Solution. cc.
Guinea-pig serum.....	0.0025	0.025
Rabbit serum.....	0.005	0.05
Rat serum.....	0.005	0.1
Goose serum.....	0.015	0.035
Chicken serum.....	0.015	0.035
Goat serum.....	0.05	no "completion"
Pigeon serum.....	no "completion"	0.035
Horse serum.....	no "completion"	no "completion"

This table again shows that the unitarian view, according to which each serum contains only a single complement, lacks all probability, for it is to be expected that in that case the zoological relationship of certain animal groups would manifest itself in their complements to a greater degree than it actually does. When, for example, we here see that the rabbit immune body is not reactivated

by horse serum but is reactivated by goose serum, we should necessarily have to conclude that "the" complement of the goose is much more closely related to "the" complement of the rabbit than is that of the horse. From the unitarian standpoint also a more marked difference should be manifested by the complements of the goose, the chicken, and the pigeon, for the first two reactivate the immune body, while the last does not. *A priori*, therefore, the unitarian view is very improbable; but aside from this the reactivating experiment with the goose immune body (which shows this to be reactivated by all three avian sera) speaks against this view.

All of these facts are readily explained if we accept the pluralistic view that each serum contains a large number of complements, and that certain types have a wide distribution in many classes of animals. In these they may be completely similar, or, what is of primary importance, their haptophore groups may be identical. It may very well be that *the avian sera are alike in the greater part of their partial complements, and that therefore all three sera may in certain cases—e.g., with the immune body of a goat immunized with ox blood—reactivate in like manner.* But it is not necessary that these three species correspond in *all* their complements, and so it may happen that a certain partial complement which is absent in pigeon serum is present in the other sera. This occurs in the above case with the immune body of the rabbit immunized with ox blood (and with that of the goat similarly treated).

I should like to emphasize one more point. The immune body of the rabbit immunized with ox blood is not reactivated by pigeon serum, whereas the immune body of the goat immunized with ox blood is thus reactivated. *This fact in itself should occasion no surprise whatever.* The tissue receptors which are present in the avian organism, and which constitute the matrix of the amboceptors in question, possess complementophile groups that fit complements widely distributed throughout the avian body. It is not at all remarkable, therefore, that the immune body obtained from the goose finds complements in various avian sera. In like manner it can readily be understood why pigeon serum is unable to reactivate the immune body of the rabbit immunized with ox blood.

The general conclusion, however, that the avian complements in their entirety are different from those of mammals, cannot be drawn from this, as is shown by the reactivation of the rabbit immune body by goose and chicken sera.

This brief analysis will show us that the complementophile groups of the immune bodies do not in general possess the great importance which we must ascribe to the cytophile groups. In order to obtain the greatest *therapeutic* effect from the immune bodies, their complementophile groups and the provision of suitable complements cannot, of course, be neglected. In this connection Dönitz (*Klin. Jahrbuch*, 1897) first pointed out the importance, in the therapy of infectious diseases, of finding sufficient sources of complements. The conditions determining this have been more closely defined by Ehrlich in his Croonian Lecture<sup>1</sup> of March 22, 1900, as can be seen from the following extract:

"Dr. Neisser at the Steglitz Institute sought to find an explanation of Sobernheim's experiments. He was able to determine that anthrax serum failed in mice even if large quantities of fresh sheep-serum (i.e., containing an excess of 'complement') were introduced at the same time. The failure in this case appears to be due, on the one hand, to the destruction, in the body of the mouse, of the 'complement' present in the sheep serum, and, on the other hand, to the fact that the 'immune body' yielded by the sheep does not find in the mouse an appropriate new 'complement.'

"From this it appears that in the therapeutic application of antibacterial sera to man, therapeutic success is only to be attained if we use either a bacteriolysin with a 'complement' which is stable in man (*homostabile complement*), or at least a bacteriolysin the immune body of which finds in human serum an appropriate 'complement.' The latter condition will be the more readily fulfilled the nearer the species employed in the immunization process is to man. Perhaps the failure which has as yet attended the employment of typhoid and cholera serum will be converted into success if the serum be derived from apes and not taken from species so distantly removed from man as the horse, goat, or dog. However this may be, the question of the provision of the appropriate 'complement' will come more and more into the foreground, for it really represents the center round which the practical advancement of the bacterial immunity must turn."

In view of the fact that every normal serum contains a great many complements, of which a larger or smaller part fits the most varied immune bodies, the need of artificially supplying complements

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<sup>1</sup> Proceedings of the Royal Society, Vol. 66.



would seem to indicate that our therapeutic efforts be directed primarily to exciting the greatest production of the organism's own complements.<sup>1</sup> The production of these complements can surely be increased by means of artificial procedures; and this is borne out by a few experiences in this direction. Thus Nolf, by injecting certain foreign sera, and P. Müller, by injecting pepton, have succeeded, in animal experiments, in increasing the production of complement. This increase may perhaps be referable to a hyperleucoeytosis in accordance with the views held by Metchnikoff and Buchner. We are certain that at least the complements originally peculiar to the organism will be able to act when fitting complementophile groups present themselves; this need not necessarily be the case, however, when foreign complements are introduced. In this question it is of no consequence whether the absence of complement action is due to destruction, to complementoid formation, or to a combination in the organism such as has been demonstrated by the ready binding of anticomplements.<sup>2</sup> The question raised by Dönitz, relative to the provision of really plentiful sources of complement, has not thus far been solved. It still remains to be seen whether the interesting investigations of Wassermann<sup>3</sup> on the completion of typhoid immune bodies with ox serum will lead to results which can be practically utilized. The amount of complement contained in the serum of the larger laboratory animals is not, as a rule, great enough to make the employment of these sera applicable for human therapeutic purposes. Wassermann, for example, found that with a method of procedure which excluded the above-mentioned diminution of complements (since he injected bacteria, immune body, and complement mixed together into the peritoneal cavity) it required 4 cc. ox serum to achieve curative results. This amount of serum in itself causes severe injury to the animals experimented upon.

*Such being the case, it seems that in the matter of supplying complements, the method suggested by us, namely, the employment of*

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<sup>1</sup>In his recent study (Zeitschrift für Hygiene, No. 37) Wassermann also lays great stress on the increase of the individual's own complements. We were especially gratified to see that in regard to the multiplicity of the complements Wassermann accepts our view completely.

<sup>2</sup>This is also supported by certain experiments of von Dungern (Münch. medicin. Wochenschrift) concerning the binding of complement by certain cells *in vitro*.

<sup>3</sup>Deutsche medizinische Wochenschrift, 1900, No. 18.



*mixed sera which contain a great many different immune bodies would prove the most effective; for with the multiplicity of the immune bodies an increase of the different complementophile groups also takes place, and thus the probability increases that the normal complements present, especially those in the human organism, can come into action most effectively.*

## IX. CONCERNING THE MODE OF ACTION OF BACTERICIDAL SERA.<sup>1</sup>

By MAX NEISSER, Member of the Institute, and Dr. FREDERICK WECHSBERG.

OUR experiences with diphtheria curative serum have taught us that in antitoxin the employment of a high dose of antitoxin is of primary importance. It is immaterial whether an excess of antitoxin is administered, since it may be regarded as certain that an excess does no harm and can on the contrary only be of benefit.

Concerning the action of bactericidal sera, however, the literature contains a number of examples which indicate that here an excess of immune serum is occasionally injurious. Thus several high authorities have published protocols of therapeutic experiments on animals which seem to contain paradoxical results; for with the same infection and varying amounts of immune serum not only those animals died which had received the smallest amounts of serum but also those which had received the largest amounts. Only those animals were protected which received doses of immune serum lying between these extremes. Such a protocol, for example, was published by Löffler and Abel<sup>2</sup> on their experiments with *bacillus coli* and a corresponding immune serum. Out of nineteen guinea-pigs which had been inoculated with the same amount of culture ( $\frac{1}{10}$  loop) and had received varying amounts of the immune serum, only six animals were protected, those which had received doses of 0.25 to 0.02 cc. Eight animals with larger doses as well as five with smaller doses of serum died.

A similar protocol is that of R. Pfeiffer,<sup>3</sup> which states that of four guinea-pigs treated with virulent cholera and a corresponding immune serum only the two animals receiving the medium doses survived.

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<sup>1</sup> Reprinted from the Münch. med. Wochenschr., 1901, No. 18.

<sup>2</sup> F. Löffler and R. Abel, Centralbl. f. Bact., 1896, Vol. 19, page 51.

<sup>3</sup> R. Pfeiffer, Zeitschr. f. Hygiene, 1895, Vol. 20, page 215.

The same phenomenon was noticed by Leclainche and Morel<sup>1</sup> in their work on the bacillus of malignant œdema, and these authors had similar experiences with erysipelas of swine and with symptomatic anthrax. As a result of this they concluded that there was a "*dosis optima neutralisans*" of the immune serum.

Since we encountered the same phenomenon in bactericidal test-tube experiments it seemed advisable to undertake a study of these occurrences, especially because the question seemed to offer points of vantage important both theoretically and practically. None of the authors above mentioned has furnished an adequate explanation of the phenomenon.

In our experiments the bactericidal action was determined in two ways, namely, with the aid of the bioscopic method previously described by us,<sup>2</sup> and by means of plate countings. The methods gave identical results even in parallel series. In order, therefore, to facilitate looking over the results we shall here give only the results obtained by the counting method.

The method of procedure was generally as follows:  $\frac{1}{5000}$  cc. of a one-day bouillon culture of the bacterium in question was put into each of a series of test-tubes. To this were added varying amounts of immune serum inactivated at 56° C. and equal amounts of the complementing active serum; or in another series, equal amounts of immune serum and varying amounts of the complementing serum. It was so arranged that all the tubes contained equal amounts of fluid, usually 2.5 cc. Dilutions were made with 0.85% salt solution. Furthermore three drops of bouillon were added to each tube, for we had convinced ourselves that this assured a good growth in the control tubes. Numerous control tests were necessary nevertheless, even if only to test the sterility of the sera employed. The specimens were kept at 37° C. for three hours and then plated on agar, using five drops from pipettes of uniform size for each plate. The results were noted by comparison and estimation, somewhat after the following scheme: 0, isolated, hundreds, thousands, infinite number.

Omitting the very extensive preliminary tests the following example is given to show the phenomenon studied by us. The immune serum employed was obtained from a rabbit by treatment

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<sup>1</sup> Leclainche and Morel, La Sérothérapie de la septicémie gangreneuse, *Annal. de l'Inst. Pasteur*, 1901, No. 1.

<sup>2</sup> *Münch. med. Wochenschr.*, 1900, No. 37.

with vibrio Metchnikoff. This serum was inactivated by heating to 57° C. for half an hour. Normal active rabbit serum served as complement.

TABLE I.

	Amount of Culture.	Inactive Immune Rabbit Serum against Vibrio Metchnikoff. cc.	Normal Active Rabbit Serum as Complement. cc.	Number of Colonies on the Plate.
	1 cc. of a one-day bouillon culture of vibrio Metchnikoff.	1.0	0.3	∞
		0.5	0.3	∞
		0.25	0.3	Many thousands
		0.1	0.3	Several hundred
		0.05	0.3	About 100
		0.025	0.3	About 50
		0.01	0.3	0
		0.005	0.3	0
		0.0025	0.3	About 100
		0.001	0.3	∞
		0.0005	0.3	∞
Control I.....		—	—	∞
“ II.....		0.01	—	∞
“ III.....		1.0	—	0
“ IV.....		—	0.3	∞
“ V.....		—	1.0	0

Three drops of bouillon to each tube. All the tubes filled to the same volume with 0.85% salt solution, then placed into the thermostat at 37° C. for three hours. Finally, five drops of each plated on agar.

This experiment shows that the inactive immune serum alone is innocuous to *vibrio Metchnikoff* (Control II); also that 0.3 cc. of the active normal rabbit serum alone is innocuous. However when, for example, 0.01 cc. immune serum is mixed with 0.3 cc. normal active rabbit serum, the many thousand germs inoculated are killed. In the same way 0.005 cc. immune serum plus 0.3 cc. normal active rabbit serum also causes the death of all the organisms. With smaller amounts of immune serum (but with the same amount of the complementing serum as before) the destruction of the germs is incomplete, while with still smaller amounts there is no destruction whatever. But the destructive effect also becomes less when more than 0.01 cc. immune serum is used, so that with 0.5 cc. immune serum no destructive at all can be observed. Hence if we had tested only the mixture of 0.5 cc. of this immune serum plus 0.3 cc. normal active rabbit serum we should certainly not have supposed that we were dealing with a powerful immune serum. That this action

is due only to the serum's content of *immune body* is shown by the following experiment in which inactive immune serum is compared with inactive normal serum of the same species, both sera being complemented with active normal serum.

TABLE II.

Amount of Culture.	Amount of the Complementing Normal, Active Rabbit-serum. cc.	Number of Colonies on a Plate on the Addition of Serum from a Rabbit Immunized against <i>Vibrio Metchnikoff</i> , the Serum having been Inactivated.				
		—	1.0 cc.	$\frac{1}{2}$ cc.	$\frac{1}{4}$ cc.	$\frac{1}{8}$ cc.
$\frac{1}{1000}$ cc. of a one-day bouillon culture of <i>vibrio Metchnikoff</i> . . . . .	1.0	$\infty$	$\infty$	a few	0	0
	$\frac{1}{2}$	$\infty$	$\infty$	many thousands	0	0
	—	—	$\infty$	$\infty$	$\infty$	$\infty$

Amount of Culture.	Amount of Normal Active Rabbit-serum. cc.	Number of Colonies on a Plate on the Addition of Inactive Normal Rabbit-serum.			
		1 cc.	$\frac{1}{2}$ cc.	$\frac{1}{4}$ cc.	$\frac{1}{8}$ cc.
$\frac{1}{1000}$ cc. of a one-day bouillon culture of <i>vibrio Metchnikoff</i> . . . . .	1.0	$\infty$	$\infty$	$\infty$	$\infty$
	$\frac{1}{2}$	$\infty$	$\infty$	$\infty$	$\infty$
	—	$\infty$	$\infty$	$\infty$	$\infty$

Control I.  $\frac{1}{1000}$  cc. bouillon culture + 2 cc. 0.85% salt sol. + 3 drops of bouillon, planted as above, result  $\infty$ .

“ II. Sterility of the immune serum, 0.

“ III. “ “ “ inactive normal rabbit-serum, 0.

“ IV. “ “ “ active normal rabbit-serum, 0.

All the tubes made up to equal volumes with 0.85% salt solution, then placed into a thermostat at 37° C. for three hours. Finally, five drops of each specimen plated on agar.

This experiment, too, shows that  $\frac{1}{16}$  cc. immune serum plus 1 cc. or  $\frac{1}{3}$  cc. normal active rabbit serum kills the germs completely; while *larger* doses of the immune serum are *less* effective. The addition of *normal* inactive rabbit serum has no effect.

The same phenomenon can be demonstrated in another manner. For the complementing serum any active serum is used which by itself already possesses a slight destructive action. If to such a serum varying amounts of an inactive immune serum are added, it will at times be found that small quantities of the latter increase

the action of the normal active serum, while somewhat larger quantities weaken the action. Still larger quantities may inhibit the action completely.

In the following experiment an immune serum was employed which had been obtained by immunizing a goat with *vibrio Nordhafen*. This serum was inactivated by heating it to 57° C. Normal active goat serum served as complement. (See Table III.)

The first column shows that normal active goat serum by itself kills bacteria, even in doses of about 0.1 cc. The fourth and fifth columns show that this bacteriolytic effect of the normal active goat-serum is in no way affected by the addition of 1.0 cc. or 0.1 cc. inactive *normal* goat serum. From the third column we see, however, that if we add to the normal active goat-serum 0.1 cc. inactive *immune serum*, the bacteriolytic effect of the former is *lowered*, and that it is almost neutralized when 1.0 cc. of the inactive immune serum is added. (Column 2.)

TABLE III.

Amount of Culture.	Amount of Complementing Normal Active Goat Serum. cc.	1	2	3	4	5
		Number of Colonies on a Plate on Addition of Inactive Goat Immune Serum against <i>Vibrio Nordhafen</i> .			Number of Colonies on a Plate on Addition of Inactive Normal Goat Serum.	
		—	1.0 cc.	0.1 cc.	1.0 cc.	0.1 cc.
$\frac{1}{100}$ cc. of a one-day bouillon culture of <i>vibrio Nordhafen</i> .	1.0	0	about 50	0	0	0
	0.5	0	many hundreds	0	0	0
	0.25	0	$\infty$	0	0	0
	0.1	0	$\infty$	several hundred	0	0
	0.05	about 50	$\infty$	$\infty$	about 10	a few
	0.025	$\infty$	$\infty$	$\infty$	$\infty$	$\infty$
	—	—	$\infty$	$\infty$	$\infty$	$\infty$

Control I.  $\frac{1}{100}$  cc. bouillon culture + 2 cc. 0.85% salt solution + 3 drops bouillon =  $\infty$ .

“ II. Sterility of the inactive immune serum, 0.

“ III. “ “ “ “ normal goat serum, 0.

“ IV. “ “ “ “ active normal goat serum, 0.

Three drops of bouillon to each tube.

All the tubes made up to equal volumes with 0.85 % salt solution.

Kept in the thermostat at 37° C. for three hours.

Finally, two drops of each specimen plated on agar.

The same phenomenon is shown by the following protocol:

TABLE IV.

Amount of Culture.	Amount of the Complementing Active Normal Guinea-pig Serum. cc.	Number of Colonies on a Plate on the Addition of <i>Inactive Goat Immune Serum</i> directed against <i>Vibrio Nordhafen</i> .			
		—	1.0 cc.	0.1 cc.	0.01 cc.
$\frac{1}{100}$ cc. of a one-day bouillon culture of vibrio Nordhafen.	1.0	0	many thousands	a few	0
	0.5	0	almost $\infty$	about 100	0
	0.25	a few	$\infty$	several hundred	a few
	0.1	several thousand	$\infty$	$\infty$	about 100
	0.05	$\infty$	$\infty$	$\infty$	many hundred
	0.025	$\infty$	$\infty$	$\infty$	$\infty$
	—	—	$\infty$	$\infty$	$\infty$

Amount of Culture.	Amount of the Complementing Active Normal Guinea-pig Serum. cc.	Number of Colonies on a plate on the Addition of <i>Inactive Normal Goat Serum</i> .		
		1.0 cc.	0.1 cc.	0.01 cc.
$\frac{1}{100}$ cc. of a one-day bouillon culture of vibrio Nordhafen	1.0	0	0	0
	0.5	about 100	0	0
	0.25	a few hundred	a few	a few
	0.1	$\infty$	a few hundred	several thous.
	0.05	$\infty$	$\infty$	$\infty$
	0.025	$\infty$	$\infty$	$\infty$
	—	$\infty$	$\infty$	$\infty$

Control I.  $\frac{1}{100}$  cc. bouillon culture + 2 cc. 0.85% salt solution + 3 drops bouillon. Result,  $\infty$ .

“ II. Sterility of the goat immune serum, 0.

“ III. “ “ “ normal goat serum, 0.

“ IV. “ “ “ “ guinea-pig serum, 0. Three drops of bouillon to each tube.

All the tubes made up to an equal volume with 0.85% salt solution.

Kept in the thermostat at 37° C. for three hours. Finally, two drops of each plated on agar.

We succeeded in obtaining similar results in such experiments with the following combinations:<sup>1</sup>

<sup>1</sup> We should like to call attention to a case which we encountered a number of times. We found that an immune serum obtained from a goat could be

Typhoid+inactive immune serum (dog)+normal active guinea-pig serum.					
Vibrio Nordhafen+inactive immune serum (rabbit)+normal active horse serum;					
"	"	"	"	"	" +normal active goat serum;
"	"	"	"	"	" serum;
"	"	"	"	"	" +normal active sheep serum;
"	"	"	"	"	" +normal active guinea-pig serum.

In order to meet the objection that the agglutinins may possibly have interfered in the experiments we have devised the following method of demonstrating the phenomenon in question:

Typhoid bacilli were subjected for one hour at 37° C to the action of inactive immune serum derived from a dog. As we know from the hæmolytic experiments of Ehrlich and Morgenroth, this results in anchoring the interbody present in the immune serum to the bacteria. The mixture was then centrifuged and the fluid poured off. After carefully shaking the sediment with a little fluid it was divided into two equal parts, to one of which inactive immune serum (dog) was added, while the other received some normal inactive dog-serum. Finally there was added to both portions the same amount of a complementing serum (normal active guinea-pig serum) which by itself was able to kill the bacteria. At the end of three hours plate cultures were made in the usual manner. The results showed that no destruction had occurred in the tube containing the excess of immune serum, whereas the culture in the other tube had been killed.

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reactivated for *Vibrio Nordhafen* by a complement derived from rabbits. In this combination we again observed the phenomenon of deflection of complement by an excess of immune body. But even *normal* inactivated goat serum (which contains interbody) when used in exactly the same amounts manifested deflection of complement. Since no quantitative difference could be discovered between the *immune serum* and the *normal* serum, we assume that in this case the deflection of complement has been effected by a substance in *normal* goat serum, as, for instance, another interbody of special affinity or perhaps a normal anticomplement. Not every complement can be used to reactivate a serum, for the complement may be deflected from the place of its intended action by any interbody, provided merely that this possesses sufficient affinity for the complement. It will be necessary to seek experimentally for combinations in which such disturbing deflections are absent and in which the difference in the affinity of the interbody which may be normally present and of that produced artificially in large quantities becomes very manifest.



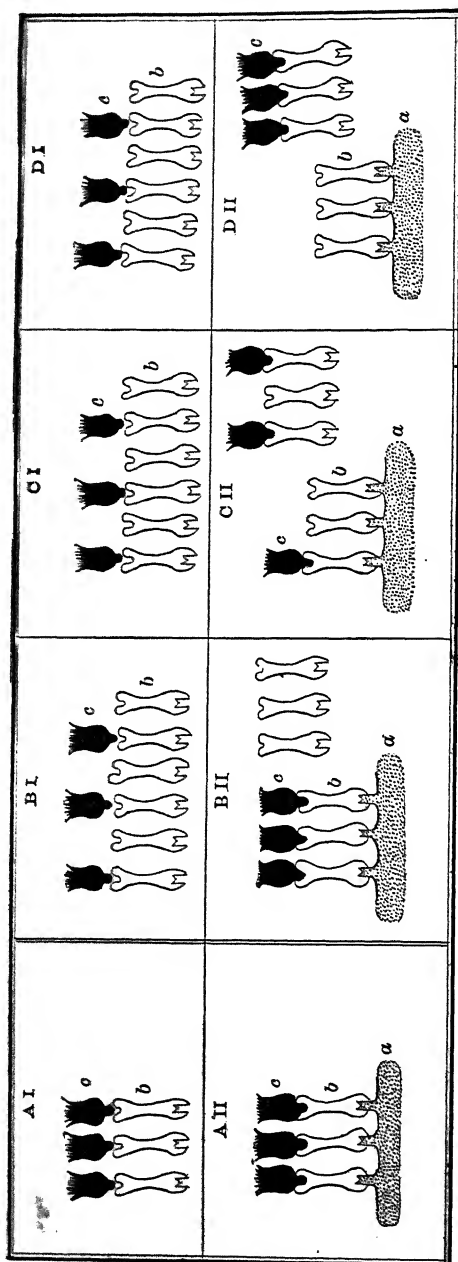
*All these experiments show that the effect produced by a given amount of complementing serum, just sufficient to reactivate a definite quantity of inactive immune serum, was diminished when large amounts of immune serum were employed. In like manner it was possible to inhibit the activity of a normal serum which was bactericidal by itself, by the addition of large amounts of the immune serum.*

It seems to us that an explanation of these important phenomena is possible only on the basis of the newer views of Ehrlich and Morgenroth. From the work of these authors on hæmolysins and from our own bacteriolytic experiments we know that the immune serum contains a thermostable interbody (amboceptor) which while itself inactive renders the complement effective by linking itself, on the one hand, to the bacterium or erythrocyte to be dissolved, and on the other to the complement. The complements, as is well known, are thermolabile and are contained in normal sera. But the interbody may also be normally present in a serum. This follows from the side-chain theory, and has already been emphasized.<sup>1</sup> An instance of this is shown in Table IV. The normal active guinea-pig serum contained complement and interbody. But besides this it contained additional complement, which became manifest when more interbody, in the form of inactive immune serum, was added. In example II it was impossible to demonstrate an interbody in the normal serum, for this by itself did not kill the bacteria even though inactive *normal* serum was added. It did, however, contain complement, and this became manifest when inactive *immune* serum was added. These phenomena are exactly like those observed with hæmolysins which have recently been so carefully studied. This one phenomenon, however, of the ineffectiveness of large doses of immune serum has not thus far been encountered in hæmolysins. This is apparently due to differences in the affinities of the interbodies, as we shall presently show.

In Fig. 1, on the next page, A II represents schematically a bacterium *a* with a number of receptors; for there are many reasons why we should assume that each bacterium possesses a number of receptors of the same kind. According to the side-chain theory, if we inject this bacterium into an animal an overproduction of the corresponding group will occur, resulting in a serum which is rich in body *b*. This body *b*, however, is not able by itself to injure the

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<sup>1</sup> Deutsche med. Wochenschr., 1900, No. 49.



bacteria, and a bacterium all of whose receptors are laden with *b* need not at all be injured in its vitality. Body *b* normally possesses a peculiar function, namely, to serve as a coupling member or link, and hence it possesses two groups (amboceptor). In this particular case one of these groups fits the receptor of the bacterium, the other possesses a peculiar relation to those normal ferment-like constituents of sera which Ehrlich has termed complements. When therefore to a normal serum which contains suitable complement we add equivalent amounts of immune serum, the condition pictured in A I will result. On adding the corresponding bacterium to this we get the condition shown in A II, in which all the bacterial receptors are occupied with immune bodies, or, more accurately, with immune bodies which on their part are loaded with bacteriolytic complement *c*. In the case here presented we shall say that it requires the occupation of all the receptors with complemented interbodies to cause the death of the bacterium.

If now to an equivalent mixture of complement and interbody we add an excess of interbody, it will be possible for only a *part* of the interbody to be loaded with complement, leaving a portion of the interbody uncomplemented. On adding the corresponding bacteria a number of conditions may result; the affinity of the interbody for the bacterial receptor may, as a result of the loading with complement, (1) remain *unchanged*, (2) it may thereby be *increased*, or (3) be *diminished*.

In the figure, B II shows the condition of increased affinity. Of the six interbodies only those combine with the bacterium which have become laden with complement. In this case therefore the excess of interbodies will have no influence on the bactericidal effect. The condition is really the same as A II, except that free interbody is also present.

C II shows the condition of unchanged affinity. In this case, if we add the bacterium to the mixture of complement and excess of interbody, all the receptors of the bacterium will, to be sure, be occupied by interbodies, but this will be entirely without any regard to the fact that these interbodies are or are not loaded with complement. It may therefore happen that only a few of the bacterial receptors will be occupied by complemented (i.e. active) interbodies, while the rest of the bacterial receptors are occupied by uncomplemented (hence inactive) interbodies. As already mentioned, however, the vitality of such a bacterium is not necessarily destroyed.

D II represents the last conceivable case. It is assumed that the "completion" of the interbody has resulted in a diminution of the latter's affinity for the bacterial receptor. In this case primarily only the uncomplemented interbodies will combine with the bacterial receptors, while the free fluid will contain complemented interbodies.

In cases C II and D II, therefore, the *excess* of interbody is not without important results; for whereas in mixtures of equivalent amounts of complement and interbody all the interbodies are complemented and so made active, the *excess* of interbody will exert a deflecting action on the complements in case C II as well as in D II, thus diminishing the end results.

The conditions shown in B II are apparently those which apply to the hæmolysins, for extensive investigations in this direction by Ehrlich and Morgenroth, concerning which we are permitted to report, have shown that deflection of complement by an excess of interbody is not observed in hæmolysis. In this case only the complemented interbodies seem primarily to be anchored by the receptors of the erythrocytes.

In the *bactericidal* sera investigated by us, however, the deflection of complement shown in C II and D II is observed, though of course we are as yet unable to say which of the two possible modes is present in any particular case. The same explanation which we have given for the phenomenon observed *in vitro* must also be held to apply to the experiments on animals, at least so far as the phenomenon above described was observed. It is perfectly obvious that when appropriate affinities of the interbody exist and when there is a marked disproportion between complement and interbody, a deflection of complement by the excess of interbody can occur in the animal body.

The phenomenon of deflection as described may perhaps present further points for study. We know that immunization causes an increase only of the interbody and that therefore every immune serum presents a deficiency of complement in comparison to interbody. Hence it is conceivable in a highly immune animal, i.e. one in which through immunization a great increase of interbody has occurred, that after infection the phenomenon of complement deflection through the excess of immune body could occur. That it actually does occur we conclude from the following statement by R. Pfeiffer:

"It has frequently happened to me that highly immunized guinea-pigs died after an injection of moderate amounts of virus. On section there were then found in the peritoneum living vibrios, sometimes even in considerable numbers. Notwithstanding this the heart blood of the cadaver when introduced into new guinea-pigs manifested the strongest power to dissolve vibrios."

It is therefore conceivable that an individual can lose its natural resistance by producing too large an amount of interbody in proportion to the amount of its complement. Such an excess of interbody then would act injuriously rather than helpfully.

This phenomenon is also of some theoretical significance. While it can readily be explained by means of the views of Ehrlich and Morgenroth, it appears, to us at least, to be absolutely irreconcilable with the theory of Bordet. This author, as is well known, regards Ehrlich's interbody as a substance capable of sensitizing the bacteria whereby they are made vulnerable to the action of the solvent "alexin" (Ehrlich's complement). If this were the case it would be absolutely incomprehensible how an excess of sensitizing substance could diminish the total effect; at the most such an excess could only increase the sensitizing action, not decrease it. Since, however, we have actually observed this decrease very frequently, we must regard this as a weighty objection to Bordet's theory.

Equally incomprehensible from Bordet's standpoint is the following observation. As has been shown above it is possible to overcome the action of an equivalent mixture of interbody and complement (the mixture itself being fatal) by adding a large excess of interbody to it. When, however, through the addition of more complement the equivalence of the mixture is again restored, the action returns. This action therefore depends not only on the *absolute* amounts of complement and interbody, but also and essentially on the *proportion* in which these two substances are present. This is true at least in the sense that not very much more interbody should be present than is required.

## X. THE DEFLECTION OF COMPLEMENTS IN BACTERICIDAL TEST-TUBE EXPERIMENTS.<sup>1</sup>

By Dr. A. LIPSTEIN, Assistant in the Bacteriological Division.

IN a study published in 1901,<sup>2</sup> Neisser and Wechsberg described a peculiar phenomenon occurring in bactericidal test-tube experiments. This phenomenon consisted in the fact that the bacteria were not killed despite the presence of the appropriate bacterial amboceptor (immune body) and complements when a comparatively large excess of amboceptor was present. This fact, for which all other explanations failed, was explained by the authors on the basis of Ehrlich and Morgenroth's views. They assumed that, with certain conditions of affinity, an excess of amboceptors exerts a deflecting and at the same time a diluting action on the complement; as a result the complement does not combine with the amboceptors anchored to the bacteria, but with the superfluous free amboceptors, while the amboceptors which are anchored to the cells remain without any complement. Now since only those complements exert a bactericidal action which are anchored to the bacteria by means of the amboceptors, it follows that in this case there will be no bactericidal action. Naturally this phenomenon of deflection of complement does not occur with every combination of amboceptor and complement, but only when certain conditions of affinity are present. Later I shall be able to show how the same amboceptor in excess exerts a deflecting action on one complement while it fails to do so on two other complements. Because of the theoretical importance of this phenomenon and its explanation, a continuation of the experiments of Neisser and Wechsberg, taking special cognizance of the objections since made, seemed desirable.

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<sup>1</sup> Reprinted from *Centralblatt für Bact.*, Vol. XXXI, No. 10, 1902.

<sup>2</sup> See page 120 of this volume; also Wechsberg, *Zeitschr. f. Hygiene*, Vol. 39, 1902.

In the following experiments the method is the same as that of the above authors, to whose work I refer for these details. The phenomenon of the deflection of complement can be exhibited in two ways: First by employing as a source of complement an active, in itself not bactericidal serum and showing that when decreasing amounts of inactive immune serum are added, only the medium amounts of the same exert a bactericidal effect, whereas both the larger and the smaller amounts are ineffective. The results shown in Table I will serve as an example of this.

TABLE I.<sup>1</sup>

Amount of Culture.	Amount of the Complementing Active Pigeon Serum.	Number of Colonies on a Plate on the Addition of Inactive Chicken Immune Serum Directed against <i>Vibrio Metchnikoff</i> .								
		1.0 cc.	0.3 cc.	0.1 cc.	0.03 cc.	0.01 cc.	0.003 cc.	0.001 cc.	0.0003 cc.	0.0001 cc.
$\frac{1}{8}$ cc. of a one-day bouillon culture of <i>vibrio Metchnikoff</i> }	0.4	$\infty$	$\infty$	$\infty$	20 to 30	0	0	0	many thousand	$\infty$

Control I.  $\frac{1}{8}$  cc. bouillon culture + 2 cc. 0.85% salt solution. Result,  $\infty$ .  
 " II. 0.4 cc. active pigeon serum +  $\frac{1}{8}$  cc. bouillon culture. Result,  $\infty$ .  
 " III. Sterility of all the sera, 0.

The second method consists in employing a serum or serum mixture which will kill the amount of bacteria employed. By adding to this decreasing amounts of inactive immune serum (or, as a control, inactive normal serum) it is found that the immune serum, in proportion to the amount added, exerts an *antibactericidal* effect, whereas the normal serum fails entirely to do this or does so in a very much less degree. This is illustrated by the results in Tables III and IV, columns 1 and 2.

In opposition to the explanation furnished by Neisser and Wechsberg, according to which the deflection of complements is caused by an *excess of amboceptors*, the following points have been raised:

- A. The phenomenon is due to agglutination of the bacterial culture;
- B. It is due to *normal* anticomplements (Metchnikoff).

<sup>1</sup> Each tube also receives three drops of bouillon as in Neisser and Wechsberg's experiments. This applies also to the rest of our experiments.

C. It is due to anticomplements which arise during the *immunizing process* (Gruber).

I shall critically examine each of these objections beginning with the first.

**A. Is the Deflection of Complements in any way Connected with Agglutination?**

This very natural objection, namely that each immune serum agglutinates the corresponding bacteria and that it is this mechanical effect of clumping which causes the bactericidal power of the immune serum to fail, Neisser and Wechsberg sought to overcome by the following method of procedure. They first agglutinated the bacteria which were to be used in the cultures, and then studied the effect that a normal and an immune serum exerted on these, with the result that a deflection of complement was obtained only with the immune serum. The following experiment also shows that the agglutinating action of an immune serum is in no way the cause of the deflection of complement; for in it I succeed in showing that an immune serum which agglutinates strongly is nevertheless unable to exert any deflecting action on the complements.

In this experiment two immune sera acting against vibrio Metchnikoff are employed, namely, that of a goose (A) and that of a goat (B). Both sera strongly agglutinate vibrio Metchnikoff, i.e., even in a dilution of 1:1000. The method of procedure is such that decreasing amounts of the inactive sera were reactivated with rabbit serum (column 1) and with pigeon serum (column 2). Now while the immune serum of the goat (B) shows a typical picture of deflection of complement, the immune serum of the goose (A), whose bactericidal power is just as strong as that of the goat serum, is unable despite this large content of amboceptor to deflect the complement. This proves that the agglutinating action and that of complement deflection are two properties of one and the same immune serum, which may exist side by side, but that agglutination in no way causes deflection of complement.

According to our view the reason why the surplus amboceptor of the goose immune serum fails in our experiment to bring about a deflection of complement is because there is not sufficient affinity between the complements of pigeon and rabbit serum on the one hand and the free amboceptor of the immune serum on the other.



By varying the experiment I have succeeded with this same immune serum in producing this phenomenon of deflection on another complement, thus furnishing evidence of the correctness of these views. The source of this complement was an active normal goat serum which in itself was bactericidal for the amount of organisms employed in the culture. (See Control II.) This experiment is otherwise similar to the preceding, except that as additional control tests the corresponding normal sera have been subjected to examination respecting their deflecting power.

TABLE II.

## A.

Amount of Culture.	Amount of In-active Goose Immune Serum Directed against Vibrio Metchnikoff. cc.	Number of Colonies on a Plate after Completion with	
		0.3 cc. Active Normal Rabbit Serum.	0.4 cc. Active Normal Pigeon Serum.
$\frac{1}{800}$ cc. of a one-day bouillon culture of vibrio Metchnikoff. ....	1.0	0	0
	0.3	0	0
	0.1	0	0
	0.03	0	0
	0.01	10	0
	0.003	many thous'd	100
	0.001	$\infty$	$\infty$

## B.

Amount of Culture.	Amount of In-active Goat Immune Serum against Vibrio Metchnikoff. cc.	Number of Colonies on a Plate on Completion with 0.3 cc. Active Normal Rabbit Serum.
$\frac{1}{800}$ cc. of a one-day bouillon culture of vibrio Metchnikoff. ....	1.0	$\infty$
	0.3	$\infty$
	0.1	0
	0.03	0
	0.01	0
	0.003	0
	0.001	0
	0.0003	$\infty$

Control I.  $\frac{1}{800}$  cc. bouillon culture + 2 cc. 0.85% salt solution =  $\infty$ .

" II. Normal active rabbit-serum 0.3 +  $\frac{1}{800}$  cc. bouillon culture =  $\infty$ .

" III. 0.4 cc. active pigeon-serum +  $\frac{1}{800}$  cc. bouillon culture =  $\infty$ .

" IV. Sterility of all the sera, 0.

TABLE III.

Amount of Culture.	Amount of the Active Normal Goat Serum, in itself Bactericidal.	Amount of the Inactive Immune and Normal Sera.	1	2	3	4
			Number of Colonies on a Plate on the Addition of the Inactive Sera here mentioned.			
			Goat Immune Serum against <i>Vibrio Metchnikoff</i>	Normal Goat Serum.	Goose Immune Serum against <i>Vibrio Metchnikoff</i>	Normal Goose Serum.
	cc.	cc.				
3/10 cc. of a one-day bouillon culture of <i>vibrio Metchnikoff</i>	0.04	1.0	$\infty$	sev'l h'n'd	$\infty$	100
		0.3	$\infty$	0	$\infty$	0
		0.1	sev'l h'n'd	0	$\infty$	0
		0.03	0	0	sev'l h'n'd	0
		0.01	0	0	0	0

Control I. 3/10 cc. bouillon culture + 2 cc. 0.85% salt solution =  $\infty$ .

" II. Normal active goat-serum 0.04 cc. + 3/10 cc. bouillon culture = 0.

" III. Sterility of all the sera = 0.

The principal difference in this as compared with the former experiment is that the goose immune serum defects the complement even more strongly than does the goat immune serum.

Thus the objection that the deflection of complements is due to agglutination has been refuted by these experiments also. The behavior of the normal sera employed as controls, whose antibacterial power even in amounts of 1.0 cc. is very slight, will be spoken of in the next section.

#### B. Is the Deflection of Complements due to Normal Anticomplements?

The deflection of complements under discussion has been ascribed by Metchnikoff<sup>1</sup> to anticytases *normally present*. This objection falls to the ground if it can be shown that the specific immune serum exhibits a constant and distinct difference in comparison to other immune sera or to various normal sera. It is entirely immaterial if the normal sera also show this phenomenon to a slight degree, e.g. Table III, columns 2 and 4. An adequate explanation of this has already been furnished by Neisser and Wechsberg, who were also the first to describe normal anticomplements. Just this *quantitative difference* between immune serum and normal serum is one

<sup>1</sup> L'Immunité dans les Maladies infectieuses, page 313.

of the postulates of Ehrlich's theory, and it is this quantitative difference which constitutes the essential point in the deflection of complements described.

The following table includes ten different goat sera, among them three bactericidal immune sera (columns 2, 3, 4), one antitoxic serum (column 5), four hæmolytic immune sera (columns 6, 7, 8, 9), and one anticomplement serum directed against the complements of horse serum (column 10). All of these have been tested as to their complement-deflecting power against vibrio Metchnikoff.

The experiment has been slightly modified from the former, for in this I made use of a mixture of 0.1 cc. active guinea-pig serum (in itself not bactericidal, Control II) plus 0.01 cc. inactive goat immune serum (against vibrio Metchnikoff). This mixture completely killed the amount of bacterial culture used, namely,  $\frac{1}{1000}$  cc. of a one-day bouillon culture of vibrio Metchnikoff (see Control II). Decreasing amounts of various inactive goat sera were added to this, as is shown in columns 1 to 10.

According to this, the Metchnikoff immune serum exerts a specific action, and it is certainly too hazardous to assume that the Metchnikoff goat used by us happened to possess an unusually large amount of normal anticomplement. Furthermore, it is possible, as I shall show later, to furnish positive proof that the deflection of complement is caused, *not* by the anticomplements, but by the amboceptors; for by removing the amboceptors it is possible to prevent the deflection. The following experiment furnishes still further evidence against Metchnikoff's view: I examined the serum of a rabbit before and after immunization with vibrio Metchnikoff and found that the normal serum was entirely inactive, whereas after eight days the immune serum of this animal caused strong deflection. In these cases, therefore, it will not do to ascribe the deflection of complements to a normal anticomplement. That normal anticomplements do occur and that they may at times simulate the phenomenon above described is, of course, possible and has already been emphasized by Neisser and Wechsberg. In such cases suitable control tests, above all the absorption method described in the next section, will guard against errors. From all this it follows that the phenomenon of complement deflection which can be observed in suitable cases is not to be ascribed to the presence of a *normal* constituent but to one produced by *immunization*.

TABLE

Amount of Culture.	Amount of the Bactericidal Serum Mixture.	Amount of the Inactivated Goat Serum. cc.	1	2	3
			Number of Colonies		
			Normal Serum.	Immune Serum against Vibrio Metchnikoff.	Immune Serum against Vibrio Nordhafen.
1/1000 cc. of a one-day bouillon culture of vibrio Metchnikoff	0.1 cc. active normal guinea-pig serum plus 0.01 cc. inactive goat immune serum against vibrio Metchnikoff	1.0	0	almost ∞	0
		0.3	0	" ∞	0
		0.1	0	" ∞	0
		0.03	0	sev'l hun'd	0
		0.01	0	0	0
					.

Control I. 1/1000 cc. bouillon culture + 2 cc. 0.5% salt solution = ∞.

" II. Active guinea-pig serum 0.1 cc. + 1/1000 cc. bouillon culture = ∞.

### C. Is the Deflection of Complements Caused by Anticomplements Developed by Immunization?

The assumption that when immunizing with bacteria, antialexins develop in the serum of the animals treated, and that these substances exert an antibactericidal and antihæmolytic action, is made by Gruber<sup>1</sup> solely for the purpose of furnishing an explanation for the phenomenon not based on Ehrlich's views. Wechsberg<sup>2</sup> has very properly pointed out that Gruber's assumption completely contradicts all our previous experiences, for then neither by active nor by passive immunization should we benefit the organism treated, but we should even injure it. The evidence on which Gruber bases this new conception consists in hæmolytic test-tube experiments in which he shows that bactericidal immune serum hinders the hæmolysis, whereas the corresponding normal serum does not do so. Wechsberg in a recent study<sup>3</sup> was never able to obtain this result, even with the same method of making the experiment as employed by Gruber. Similar negative results were obtained by H. Sachs of this institute, who studied a number of immune sera for this purpose, viz., immune sera against vibrio Metchnikoff, vibrio Nordhafen, staphylococcus,

<sup>1</sup> Wiener klin. Wochenschr., 1901, No. 50.

<sup>2</sup> Ibid.

<sup>3</sup> Ibid., 1902, No. 13.

## IV.

4	5	6	7	8	9	10
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on a Plate on the Addition of the Inactive Goat Sera here mentioned.

Immune Serum against Staphylococcus Aureus.	Serum against Staphylococcus Toxin.	Serum against Rabbit Blood-cells.	Serum against Sheep Blood-cells.	Serum against Ox Blood-cells.	Serum against Human Blood-cells.	Serum against Horse Serum.
0	sev'l hun'd	0	20-40	many thou.	almost ∞	100
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0

Control III. Active guinea-pig serum 0.1 cc. + 0.01 cc. inactive goat immune serum against vibrio Metchnikoff +  $\frac{1}{1000}$  cc. bouillon culture.

" IV. Sterility of all the sera = 0.

erysipelas of swine, hog cholera, dysentery. I am unable to say what the cause of these contradictory results may be. One thing, however, is shown thereby, namely, that there is no general law such as Gruber assumes, and that, their toxicity taken for granted, his experiments constitute rather a rare exception which must even be regarded as an unfortunate coincidence.

That immunization with any kind of bacteria does not cause the formation of anticomplements with a *general antibactericidal* action, in Gruber's conception is seen by glancing at Table IV, columns 2, 3, and 4. In these experiments only the Metchnikoff immune serum exerted a complement-deflecting action, not, however, the immune sera of two other goats immunized with *vibrio Nordhagen*, and with *staphylococcus pyogenes aureus*.

It is very easy to prove that in the Metchnikoff immune serum the active factor which effects this anticomplementary action and which develops as a result of the immunization is really an amboceptor; for by previously adding the corresponding dead bacteria to the immune serum and later centrifuging, the amboceptor of the serum is abstracted. It can then be shown that this amboceptor-free immune serum has lost all its power to deflect complement, provided, of course, a sufficient amount of bacteria was used. It can further be shown in this way that the action proceeds quantitatively. Thus if decreasing amounts of bacteria are employed

to absorb the amboceptors, e.g., 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$  agar culture, it will be found, for instance, that the whole agar culture completely abstracts the amboceptor from the serum; one-quarter of the culture abstracts only part of the same, smaller amounts still less corresponding to the amount of bacteria added.

But by this method I was able to bring further proof of the *specific* action of the immune serum. Thus when I added dead Metchnikoff vibrios to the Metchnikoff immune serum, I was able to remove the amboceptor; the serum then did not show even a trace of deflecting action. If, however, to this Metchnikoff immune serum I added *other* bacteria (vibrio Nordhafen, typhoid, dysentery) the serum lost none of its power to deflect complement, because the immune body of Metchnikoff immune serum is anchored only by Metchnikoff vibrios, and not by any other kind of bacteria.

Such an experiment is reproduced *in extenso* below. For certain reasons to be explained directly, this requires a tedious and complex method of procedure. As in the previous experiment I employed a mixture of active guinea-pig serum and inactive Metchnikoff immune serum (from a goat), which sufficed to kill the amount of bacterial culture employed (Control II). To this mixture are added decreasing amounts of the native inactive Metchnikoff immune serum of a goat (column 1). The same immune serum previously treated with Metchnikoff vibrios is shown in column 2; treated with Nordhafen vibrios, column 3; with typhoid bacilli, column 4; and with dysentery bacilli, column 5. This preliminary treatment with bacteria is as follows: Agar cultures of the various bacteria are suspended each in 2 cc. 0.85% salt solution and killed by heating these suspensions to 65°-70° for one hour. If to these four suspensions we were now to add Metchnikoff immune serum with the object of having the immune body absorbed, we should later, on centrifuging to remove the bacteria, encounter great difficulties, it being impossible in this way to obtain a clear fluid free from bacteria. The Metchnikoff vibrios alone are an exception, because they are agglutinated by the corresponding immune serum. Although, according to Gruber, even a considerable accumulation of bacteria is without effect on hæmolysis, in my bactericidal experiments I met with the annoying fact that such large amounts of bacteria (the centrifuged fluid is cloudy) are in themselves strongly antibactericidal. I was able to overcome this difficulty as follows: 2.0 cc. of the corresponding inactive immune serum were added to the suspended agar cultures

in order to effect agglutination; thus Metchnikoff vibrios to Metchnikoff immune serum, Nordhafen vibrios to Nordhafen immune serum, etc. The mixtures were kept at 37° C. for one hour, diluted to 25 cc. with salt solution (in order to dilute the serum as much as possible), and then centrifuged. The fluids were poured off; the sediments consisting of agglutinated bacteria were thoroughly shaken, each with 2½ cc. inactive Metchnikoff immune serum and allowed to stand for 1½ to 2 hours at 37° C., the mixtures being occasionally shaken. On then again centrifuging for a long time, I was able to pour off a clear bacterial-free fluid which was used for the following experiment (columns 2 to 5).

TABLE V.

Amount of Culture.	Amount of the Bactericidal Serum Mixture.	Amount of the Inactive Metchnikoff Immune Serum.	1	2	3	4	5
			Number of Colonies on a Plate on the Addition of Inactive Goat Serum against Vibrio Metchnikoff.				
			In the Native State.	Previously Treated with Dead and Agglutinated			
				Metchnikoff Vibrios.	Nordhafen Vibrios.	Typhoid Bacilli.	Dysentery Bacilli.
100 cc. of a one-day bouillon culture of vibrio Metchnikoff	0.1 cc. active guinea-pig serum plus 0.01 cc. inactive goat immune serum against vibrio Metchnikoff	1.0	8	0	∞	∞	8
		0.5	8	0	∞	∞	8
		0.25	8	0	many thou'd	many thou'd	100
		0.1	10-20	0	10-20	10-20	0
		0.05	0	0	0	0	0

Control I. 100 cc. bouillon culture + 2 cc. 0.85% salt solution = ∞.

“ II. 0.1 cc. active guinea-pig serum + 0.01 cc. inactive goat immune serum against vibrio Metchnikoff + 100 cc. bouillon culture = 0.

“ III. Sterility of all the sera = 0.

This experiment shows that by previously adding dead bacteria of the corresponding species and then using the centrifuged serum, it is possible to remove the property by means of which an immune serum when in excess can exert a complement-deflecting action. This absorption, however, did not succeed with three other species of bacteria. Hence we can conclude that the deflecting agent of the immune serum is a substance produced by immunization and

related to the complement on the one hand (complement deflection!) and to the corresponding bacterium on the other (specific absorption). It is therefore an amboceptor in Ehrlich's sense, and not an anti-alexin in that of Gruber.

The results of my experiments may be summarized as follows:

(1) By comparing two bactericidal immune sera both possessing a strong agglutinating property, while, in certain combinations, only one manifested the phenomenon of deflection of complement, the objection was controverted that this deflection is due to the mechanical action of agglutination.

(2) It was possible to show in several different ways that the deflection of complement is not caused by a constituent of normal serum.

(3) It was directly proven that the deflecting agent of the immune serum is the specific amboceptor (immune body) produced by immunization.

From this it follows that the amboceptor merely plays the rôle of a coupling element between bacteria and complement and that the property of "sensitizing" (Bordet) or of "preparing" (Gruber) cannot be ascribed to it. The latter assumptions seem to be irreconcilable with the phenomenon of deflection of complements described by Neisser and Wechsberg.



## XI. ACTIVE IMMUNITY AND OVERNEUTRALIZED DIPHTHERIA TOXINS.<sup>1</sup>

By Dr. JULES REHNS.

INSTEAD of following the classical method of immunizing against diphtheria, namely, by inoculating the toxin in gradually increasing doses, a number of workers have attempted to produce immunity by inoculating, either from the outset or during the course of the immunizing process, mixtures in which the toxin was partly, wholly, or over neutralized. It will at once be realized that these methods, with which the names Babes, Pavlovsky, Arloing, Madsen, and Kretz are principally associated, possess an entirely different significance.

Under the direction of Professor Ehrlich I have tried to see whether active immunity could be conferred upon a given normal organism by the injection of increasing doses of diphtheria toxin mixed with one or more times its equivalent of antitoxin.

Rabbits weighing about 2000 grams were used and these were injected with mixtures composed of a toxin L and the Standard Serum of the Institute.

The constants of this poison, determined according to the classical methods devised by Ehrlich were as follows:

(1) The amount of poison which just corresponded to an immunizing unit, i.e., the limit of no action whatever,

$$L_0 = 0.3 \text{ cc.}$$

(2) The amount of poison which, mixed with one immunizing unit of serum, was just sufficient to kill the animal, the so-called  $L_+$  dose,

$$L_+ = 0.45 \text{ cc.}$$

(3) The fatal dose for a rabbit weighing about 2000 grams, death occurring in four days:

This was about 0.01.

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<sup>1</sup> Reprinted from *Compt. rend. de la Soc. de Biologie*, 1901, page 141.

Two rabbits were inoculated intravenously, one with  
3 units serum + 0.3 cc. toxin,  
a mixture neutralized about three fold; the other with  
3 units serum + 0.45 cc. toxin,  
a mixture about doubly neutralized.

The animals received daily increasing doses from the 5th to the 18th of December, 1900, at the end of which time the total amount of toxin they had received was

for the one 7.5 cc. or 750 fatal doses,  
for the other 4.27 cc. or 429 fatal doses.

The animals showed no change in health and lost no weight.

In order to allow the excess of serum introduced time to be eliminated, four weeks were allowed to elapse before testing the serum for its antitoxic strength.

A control rabbit treated with serum alone died accidentally, but, as will be seen from the results of the experiment, a control was superfluous.

Both rabbits were killed Jan. 24, 1901, and 1 cc. of the serum was mixed with one-quarter a  $L_+$  dose. The test animals died in twenty-four hours. By decreasing the quantity of toxin to one-eighth  $L_+$  dose, death occurred in forty eight-hours.

From this we see that the serum of these animals certainly contains no more than one-eighth of an immunizing unit, an amount which at once eliminates any idea of a passive immunity.

One must therefore conclude that the organism of a normal rabbit *not sensitized through previous immunization* is unable to break up the combination of diphtheria toxin with antitoxin. Not a trace of this toxin is free at any moment, and the strongest doses of the mixture are destitute of any injurious effects. Twenty fatal doses, for instance, were given at the beginning. But we see further that these mixtures do not cause even the slightest production of antitoxin. We must therefore conclude, with Arloing, that the injection of overneutralized toxin is absolutely useless for purposes of immunization.

These results do not in the least resemble those of other authors who have used *partially* neutralized mixtures in which toxins and toxonoids are present in a free state. So far as immunizing power

is concerned, Madsen has found that these substances, though absolutely free from pathogenic action, are entirely equal to pure toxin. In these, then, toxicity and immunizing power are entirely unassociated. These facts make Ehrlich's hypothesis very plausible, according to which the toxin molecule contains separate groups, "haptophore" and "toxophore." The combination of the former with corresponding groups in the receptive organs furnishes the conditions necessary and sufficient for the production of antitoxin by these organs.

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TRANSLATOR'S NOTE.—Park and Atkinson report quite different results in a similar set of experiments. By treating horses with toxin neutralized threefold (for guinea-pigs), they produced a considerable amount of antitoxin. Even when the toxin was neutralized sixfold there was a slight production of antitoxin. See Proceedings of the New York Pathological Society, 1903.

## XII. IS IT POSSIBLE BY INJECTING AGGLUTINATED TYPHOID BACILLI TO CAUSE THE PRODUCTION OF AN AGGLUTININ?<sup>1</sup>

By Prof. M. NEISSER, Member of the Institute, and Dr. R. LUBOWSKI, formerly  
Assistant in the Bacteriological Division.

ESPECIALLY important for Ehrlich's conception of the chemical union of toxin and antitoxin are the experiments in which immunization of animals was attempted with neutral, and therefore non-poisonous, toxin-antitoxin mixtures. Such experiments, inaugurated by Babes, were recently published by Kretz,<sup>2</sup> among others. At first it appeared to this author that he could really immunize with such neutral mixtures, but exact reexamination convinced him of the contrary. Jules Rehns<sup>3</sup> also was unable to obtain any results with neutralized toxin-antitoxin mixtures. All of these experiments showed that Ehrlich's conception, that of a chemical union of toxin and antitoxin, most readily sufficed to explain the facts.

In immunization with *cellular material* the circumstances are far more complex. von Dungern<sup>4</sup> therefore first attempted to rule out the immunizing action of the injected cells (erythrocytes) by simultaneously injecting the corresponding immune serum obtained elsewhere. This mixture, therefore, was neutral, and caused no immunity reaction. Our colleague, Dr. Sachs, has continued these researches at the suggestion of Professor Shield, and will report thereon the following article.

In direct contrast to v. Dungern's experiments are the results

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<sup>1</sup> Reprint from the Centralblatt f. Bacteriologie Parasitenkunde und Infektions Krankheiten, Vol. XXX, 1901, No. 13.

<sup>2</sup> R. Kretz, Ueber die Beziehungen von Toxin und Antitoxin, Zeitschr. f. Heilkunde, 1901, No. 4.

<sup>3</sup> See pages 143 et seq.

<sup>4</sup> See pages 36 et seq.

obtained by Jules Rehns<sup>1</sup> by the injection of agglutinated typhoid bacilli. He found that it was immaterial, so far as effect was concerned, whether he injected the typhoid bacilli agglutinated or not agglutinated. An entirely similar experiment has also been published by Nicolle and Trénell.<sup>2</sup>

Having previously and independently of Rehns busied ourselves with this question, and having seen that it is attended with considerable experimental difficulties, we again took up the problem on the publication of Rehns' article, especially because of the theoretical importance of the subject. Furthermore, our previous experiences had given us the impression that Rehns' results were not generally applicable.

The technique of our experiments was as follows: The typhoid culture employed was an old laboratory culture especially adapted to agglutination experiments. Of this, one-day agar cultures, suspended in physiological salt solution and killed by exposure for one hour to 60°-70° C., were used for the injections.

The preparation of the agglutinated typhoid bacilli was most carefully attended to, it being deemed especially important to fully satisfy the bacilli with the agglutinin. The agglutinin was a highly active typhoid agglutinin derived from a horse, and agglutinated even in dilutions of 1:50,000; only in the last experiments was a weaker serum used. The agglutinin was added to the bacteria in such amounts that about 500-1000 times the amount calculated to be necessary was used. In order to effect as firm a union as possible between bacilli and agglutinin the latter was allowed to act on the bacilli for one hour at 42°-44° C., during which time the tubes were shaken every ten minutes (at times with glass beads) in order to loosen the larger clumps and secure the penetration of the agglutinin to the central portions of the clumps. And in order to be on the safe side, we centrifuged the bacteria from the first mixture and repeated the saturating process in the same manner. After the second saturation the mixture was again centrifuged, filled up with salt solution, again centrifuged, and then washed several times. The various decantations were saved and tested for the presence of agglutinin; the last washings had to be free from agglutinin.

Concerning the amount of injected bacilli in conformity to our

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<sup>1</sup> J. Rehns, *Compt. rend. de la Soc. de Biol.*, 1900, page 1058.

<sup>2</sup> Nicolle et Trénell, *Compt. rend. de la Soc. de Biol.*, 1900, page 1088.

previous experience we took two agar cultures to be the normal measure for one rabbit. Since small amounts of bacilli were lost through the centrifuging, we often employed somewhat larger amounts for the injection of the agglutinated bacilli; while, on the other hand, the control animals frequently purposely received less than two agar cultures. This was done to meet the objection that the animals injected with agglutinated bacilli had received fewer bacilli than the control animals. But just in these control animals which therefore received different amounts it was seen that a strict parallelism between the amount of bacilli injected and the agglutinating value produced thereby does not exist. Many animals with smaller doses exhibited higher agglutinin values than other animals with larger doses, as is seen by the following table I.

TABLE I.

Number of the Animal.	Agglutinating Value of the Serum Previous to Injection.	Amount Injected.	
119	0	$\frac{1}{2}$ mass culture subcutan.	1:160
118	1:40	$\frac{1}{2}$ " " "	1:1280
117	1:40	$\frac{1}{8}$ " " "	1:320
159	0	$\frac{1}{2}$ " " + $\frac{1}{2}$ agar culture	1:1280
160	1:40	$\frac{1}{8}$ " " + $\frac{1}{2}$ " "	1:1280
162	0	$\frac{1}{4}$ " " + $\frac{1}{2}$ " "	1:2560

The injection was usually subcutaneous, a few times intraperitoneal. The blood was abstracted from the ear vein.

Testing the agglutinating value of the serum was accomplished according to a method long in use in the bacteriological division, as follows:

The serum dilutions (in 0.85% salt solution) were usually  $\frac{1}{20}$ ,  $\frac{1}{40}$ ,  $\frac{1}{80}$ ,  $\frac{1}{160}$ , etc.; finer gradations were not employed, as they are of no value in measuring the agglutination. The culture used was a living 20-hour agar culture which was suspended in 10 cc. of bouillon. To each serum dilution, whose volume was 1 cc., the same amount of bacilli was added (1 cc. bouillon culture), so that the total volume of each specimen was 2 cc. Each specimen was then poured into a little Petri dish and placed into the thermostat for two hours. Thereupon the specimens were examined with the low power of the dry objectives. In this way the occurrence of larger or smaller clumps is very distinctly seen. In the protocols

only "complete agglutination" and the "positively distinct agglutination" are regarded as positive; everything at all doubtful is regarded as not agglutinated.

The first question was on which day following the injection the maximum agglutinating value was to be expected in the serum of the animals. Table II gives a résumé of eight animals injected with dead typhoid bacilli and examined at different times.

TABLE II.

Number of the Animal.	Agglutinating Value of the Serum Previous to the Injection	Injected Amount.	Agglutinating Value on the				
			5th Day	7th Day	9th, 10th, or 11th Day	14th Day	15th Day
117	1:40	$\frac{1}{10}$ mass culture subcut.		1:80		1:320	
118	1:40	$\frac{1}{10}$ " " "		1:320		1:1280	
119	0	$\frac{1}{10}$ " " "		1:80		1:160	
134	1:160	2 agar cultures	1:320		1:640		
132	0	$\frac{1}{2}$ " " "	1:640		1:640		
110	?	$\frac{1}{2}$ mass culture + $\frac{1}{2}$ agar culture	1:640		1:320		1:160
109		$\frac{1}{2}$ mass culture + $\frac{1}{2}$ agar culture	1:2560		1:1280		1:320
108		$\frac{1}{2}$ mass culture + $\frac{1}{2}$ agar culture	1:1280		1:640		1:640

Four of these animals exhibited a lower value on the 7th (or on the 5th) day than on the 14th (or on the 10th) day. The other four animals showed a decrease or no change at all in their agglutinating values on the 5th, 9th, and 14th (or 5th and 10th) days. Hence if on the 7th day we examined, as we actually did, the animals which had been injected only with dead typhoid bacilli, we were not sure that we should strike the maximum agglutinating value. That we chose this time nevertheless is explained by the fact that this simplified the investigations, and by the further consideration that we did not need the highest possible values in these control animals.

The animals, however, in which we were compelled to strike the maximum value are seen by reference to Table III to have behaved differently. Of 15 animals which had been injected with dead, *agglutinated* typhoid bacilli, there were only 3 which still showed a slight increase of agglutinating value from the 7th to the 14th (or 5th-9th) day. We were therefore justified in withdrawing the

blood for examination of all the animals on the 7th day after the last injection.

TABLE III.

Number of the Animal.		Value on						
		5th Day	7th Day	9th Day	11th Day	13th Day	14th Day	15th Day
114	These all received agglutinated typhoid bacilli		1:80				1:160	
115			1:80				1:160	
111		1:80		1:160				1:80
103		1:160		1:160				1:160
104		1:80		1:80				1:80
106		1:160		1:160				1:160
132		0			0			
181			0			0		
182			1:40				1:40	
112		1:160		1:160				1:160
116			0				0	
131		0			0			
133		0			0			
161			1:20					0
105		1:160		1:80				1:80

It may further be mentioned that examinations were also made on the 29th and 39th day after the injection, in which however a decrease of the agglutinating value was usually found.

Investigations also showed that injections of physiological salt solution in bouillon caused no variation in the normal agglutinating values.

A further question was whether and to what degree the serum of normal untreated rabbits possesses agglutinating properties on typhoid bacilli. Out of 17 rabbits which were examined for this purpose, 10 showed no agglutination in dilutions of 1:20, one serum agglutinated in the dilution 1:20, but no higher, 5 others in 1:40, but no higher, and only one agglutinated even in a dilution of 1:160. (See Table IV.)

It is therefore a rare exception for normal rabbit serum to still manifest agglutinating powers on typhoid bacilli in a higher dilution than 1:40. It should be remarked that in the above table "0" has always then been put down when the agglutinating value of the serum in a dilution of 1:20=0; for the examinations began with this dilution.



TABLE IV.  
AGGLUTINATING VALUES OF NORMAL RABBIT SERUM.

Number of the Animal.	Dilution of the Serum.				
	1:20.	1:40.	1:80.	1:160.	1:320.
133	0	0	0	0	0
132	0	0	0	0	0
136	0	0	0	0	0
164	0	0	0	0	0
181	0	0	0	0	0
163	0	0	0	0	0
166	0	0	0	0	0
165	0	0	0	0	0
159	0	0	0	0	0
162	0	0	0	0	0
161	+	0	0	0	0
114	+	+	0	0	0
160	+	+	0	0	0
182	+	+	0	0	0
117	+	+	0	0	0
118	+	+	0	0	0
134	+	+	+	+	0

We now come to the experiments proper. In the first of these (Table V) a series of rabbits was injected with *agglutinated* typhoid bacilli, while a control series was injected with the same or smaller amounts of *non-agglutinated* bacilli. This comparison shows a far higher agglutinating value of the serum of the control animals than that of the other animals.

TABLE V.

Num-ber of the Animal.	Agglu-tinating Value of the Serum previous to the In-jection.		Injection of	Maximum Aggluti-nating Value.	Average.
111	?	Agglutinated typhoid bacilli	$\frac{1}{2}$ mass culture + $\frac{1}{2}$ agar culture	1:160	1:147
112	?		ditto	1:160	
103	?		ditto	1:160	
104	?		ditto	1:80	
105	?		ditto	1:160	
106	?		ditto	1:160	
108	?	Non-aggluti-nated typhoid bacilli	ditto	1:1280	1:1493
109	?		ditto	1:2560	
110	?		$\frac{1}{4}$ mass culture + $\frac{1}{4}$ agar culture	1:640	

The next question was whether rabbits really react at all to injections of agglutinated typhoid bacilli; in other words, whether the normal agglutinating value possibly present is at all increased by injections of agglutinated typhoid bacilli. The result was surprising, as is seen in Table VI. For while in four animals no increase occurred, in two others there was a very slight increase, and in four more the increase, though distinct, was insignificant in comparison with that in six animals injected with non-agglutinated typhoid.

TABLE VI.

Number of the Animal.	Agglutinating Value of the Serum previous to the Injection.	Injection of	Maximum Agglutinating Value after the Injection.	Average.
132	0	Agglutinated typhoid bacilli	2 agar cultures (intraperitoneally)	0
181	0		2 agar cultures	0
116	0		$\frac{1}{8}$ mass culture	0
182	1:40		ditto	1:40
164	0		$\frac{1}{8}$ mass culture + $\frac{1}{2}$ agar culture	1:20
163	0		ditto	1:40
166	0		ditto	1:320
115	0		$\frac{1}{8}$ mass culture	1:160
161	1:20		$\frac{1}{8}$ mass culture + $\frac{1}{2}$ agar culture	1:320
114	1:40		$\frac{1}{8}$ mass culture	1:160
165	0	Not agglutinated	$\frac{1}{8}$ mass culture + $\frac{1}{2}$ agar culture	1:640
159	0		$\frac{1}{8}$ " " + $\frac{1}{2}$ " "	1:1280
162	0		$\frac{1}{8}$ " " + $\frac{1}{2}$ " "	1:2560
119	0		$\frac{1}{8}$ " " " "	1:160
136	0		1 agar culture	1:640
160	1:40		$\frac{1}{8}$ mass culture + $\frac{1}{2}$ agar culture	1:1280

NOTE.—1 mass culture equals about 12 agar cultures.

With this the main portion of the question had been answered; for these experiments already showed that the injection of agglutinated typhoid bacilli exerts an action which quantitatively is different from that following the injection of non-agglutinated bacilli. Nevertheless even the agglutinated bacilli, although their injection is often wholly without effect, in many cases still exert a stimulus on the formation of agglutinins even though in a slight degree. This is due to individual peculiarities of the animals employed, and these we have not thus far been able to recognize in advance. The natural assumption that animals which already normally possess agglutinins react more readily to the injection of agglutinated typhoid bacilli

than do those which do not normally possess agglutinins has not been confirmed, for out of seven animals (Table VI) in whose serum no typhoid agglutinin could be demonstrated previous to treatment, three did not react to the injection of agglutinated typhoid bacilli, two reacted feebly and two very distinctly. On the other hand, out of three animals in which, previous to treatment, a typhoid agglutinin could be demonstrated, two reacted distinctly to the injection of agglutinated bacilli and one not at all.

Another assumption was, that in the animals which had reacted but feebly or not at all, an increase of the sensitiveness against agglutinated bacilli could be brought about artificially by repeated injections of agglutinated bacilli. This also has not been confirmed. Thus three animals (Table VII) reacted to the second injection of agglutinated bacilli just as little as they did to the first, one animal reacted feebly, as it had done previously, and only two animals (Nos. 131 and 133), which had failed to react to the first injection, reacted distinctly to the second. The protocols of these last two animals, however, point out a peculiarity. On the first occasion these animals were injected intraperitoneally and it is noted that at this time the intestine was pricked. The first injection may therefore have mostly gone into the bowel and so produced no effect. The second injection would then have really been the only effective one. These two cases cannot therefore be used to prove that by means of a previous injection of agglutinated bacilli an artificial increase of the sensitiveness against a subsequent injection of agglutinated bacilli can be effected. The previous injection of agglutinated bacilli, however, in no way influences the sensitiveness against non-agglutinated bacilli, as is shown by the four control animals (Table VII).

Finally experiments were made regarding still another assumption. It was conceivable that the previous injection of a certain amount of non-agglutinated bacilli would have sufficed to bring about a sensitiveness against a subsequent inoculation with agglutinated bacilli. This assumption also has not been borne out. Out of five animals (Table VIII) which, after a previous injection of non-agglutinated typhoid, received an injection of agglutinated typhoid, two showed a slight increase and three no increase in agglutinating value.

It follows from all these experiments that there is a distinct difference between the injection of agglutinated and of non-agglutinated typhoid bacilli. The injection of non-agglutinated typhoid bacilli is always followed by an increase of the agglutinating power. This

TABLE VII

Number of the Ant.	Agglutinating Power of the Serum prior to the First Injection	First Injection of	Maximum Agglutinating Value after the First Injection.	Agglutinating Value previous to the Second Injection.	Second Injection of	Number of Days after the First Injection.	Maximum Agglutinating Value thereafter.	Average.
181	0	2 agar cultures	0	0	2 agar cultures	16	0	1:117
182	0	2 agar cultures (intrapertitoneal)	0	0	1 mass culture (subcutan)	15	0	
183	1:40	2 " " " "	1:40	1:40	2 agar cultures	16	1:40	
184	0	2 " " " " intrapertitoneal	0	0	1 mass culture	16	1:320	
185	0	2 " " " " "	0	0	1 " " "	16	1:320	1:180
186	0	1 mass culture + 1 agar culture	1:20	0	2 agar cultures	21	1:20	
187	0	1 mass culture + 1 agar culture	1:160	1:80	1 mass culture	21	1:180	
188	0	ditto	1:160	1:160	1 " " "	21	1:640	
189	0	ditto	1:160	1:80	1 mass culture	21	1:640	1:180
190	0	ditto	1:160	1:160	1 " " "	21	1:640	

TABLE VIII

Number of the Animal.	Agglutinating Power before Treatment.	Had already been injected with	Agglutinating Value immediately previous to the following Injection.	Injected with	Maximum Agglutinating Value after this.	Agglutinating Power just previous to the following Injection.	Injected with	Maximum Agglutinating Value after this.
134	1:100	—	1:100	2 agar cultures	1:640	1:640	1/4 mass culture	1:1280
132	0	Twice with aggl. typhoid (See Table VII)	0	1 " "	1:640	1:640	2 agar cultures	1:320
164	0	ditto	1:20	2 " "	1:640	1:80	2 " "	1:160
181	0	ditto	0	2 " "	1:1024	1:640	2 " "	1:320
182	1:40	ditto	1:40	2 " "	1:320	1:320	2 " "	1:320

increase is usually very great and only rarely slight. The injection of agglutinated typhoid bacilli, provided that attention is paid to a sufficient saturation with agglutinin, is frequently followed by no reaction, often by a slight reaction, and rarely by a marked increase of the agglutinating value. This reacting power depends on the individuality of the animal and stands in no relation to the original agglutinating value, nor can it be influenced artificially. Furthermore, as we learned from a special experiment, it is immaterial whether the immune serum used to agglutinate the typhoid bacilli is derived from the same or from another animal species.

The explanation of these facts is not difficult provided one proceeds on Ehrlich's theory. According to this the agglutinin consists of thrust-off cell-receptors. As a result of their seizure by the bacterial receptors they have been produced in excess and give off to the circulation. They, therefore, possess a definite relation to the corresponding bacterial receptors. Hence when we fully saturate typhoid bacilli with agglutinin, we cause the bacterial receptors to be occupied, and are then as little able to cause a reaction with these bacteria as we are to cut with a sword in its scabbard.

If then, in spite of this, certain animals react to such "occupied" typhoid bacilli, we shall have to assume that these animals possess the power to dissolve the combination of agglutinin and bacterial receptor and thus set the latter free.

This action, however, never proceeds to the full extent.

Incomparably more important, and, as it appears to us, explicable only with the aid of Ehrlich's chemical views, is the main phenomenon, that in many animals no reaction whatever follows the inoculation of agglutinated typhoid bacilli; that therefore in many cases it is possible to dispose of the bacterial group giving rise to the agglutinin, by causing this group to be occupied by the corresponding agglutinin.

#### SUBSEQUENT NOTE.

R. Pfeiffer and Friedberger,<sup>1</sup> through recent experiments on cholera vibrios and cholera amboceptors, have obtained results which are in gratifying accord with those obtained by v. Dungern, M. Neisser and Lubowski, and Sachs.<sup>2</sup> In earlier experiments R. Pfeiffer<sup>3</sup> had found that the bacterial substance dissolved in the peritoneum through the influence of the cholera immune serum

<sup>1</sup> R. Pfeiffer u. Friedberger, Berl. klin. Wochenschr., 1902, No. 23.

<sup>2</sup> See the following article.

<sup>3</sup> R. Pfeiffer, Deutsche med. Wochenschr., 1901, Nos. 50-51.

usually still excited an extraordinarily strong immunity reaction, a phenomenon seemingly in contradiction to Ehrlich's theory. Further experiments, however, showed that when very high doses of an active cholera goat serum were employed, the immunizing action was almost entirely lost. Of especial importance for future methodical investigations of this kind is the fact determined by these authors, that a real saturation of the receptors of the cholera vibrios requires a surprisingly high multiple of the amount of immune serum sufficient to dissolve the same amount of cholera vibrios. 7500 times this amount does not yet satisfy all the affinities and it requires enormous doses, up to 3-4 million times, to completely saturate all the receptors.

### XIII. IMMUNIZING EXPERIMENTS WITH ERYTHROCYTES LADEN WITH IMMUNE BODY.<sup>1</sup>

By Dr. HANS SACHS, Assistant at the Institute.

THE interesting experiments of v. Dungern<sup>2</sup> have furnished further proof that the same group (receptor) of the blood-cells which in hæmolysis combines with the specific immune body causes the production of this immune body within the organism. v. Dungern injected rabbits with ox blood to which a plentiful amount of an immune body (obtained from rabbits by immunizing with ox blood) had been added, and found, as was to be expected, on the basis of the side-chain theory, that animals so treated failed to produce any immune body whatever.

The results of the investigations of M. Neisser and Lubowski<sup>3</sup> show that the complete inactivity of such saturated receptors—agglutinated typhoid bacilli—in the animal body is not at all a general rule, but that, on the contrary, a moderate development of the immunity reaction occurs even with such mixtures and that this depends on certain individual differences. Hence at the suggestion of Prof. Ehrlich I have extended the experiments of v. Dungern and undertaken blood-immunization experiments on a large series of animals. The results obtained lead to certain modifications of von Dungern's conclusions.

The method of these experiments must be guided by two principles. To begin, it is important that the receptors of the injected blood are *really saturated*, for even a very slight free residue might effect an immunity reaction in the animal body. And yet it is essential to *remove any possible excess of immune body*, because this

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<sup>1</sup> Reprint from the Centralblatt für Bacteriologie, Parasitenkunde und Infektion Krankheiten, Vol. XXX, 1901, No. 13.

<sup>2</sup> v. Dungern, Muench. med. Wochenschr., 1900, No. 20. See also page 56.

<sup>3</sup> See the preceding article, page 146.



could passively reappear in the serum of the injected animal and so simulate an active new formation of immune body. In accordance with this the experiments were made as follows: Ox blood was treated with an excess of inactive serum from a rabbit which had been immunized with ox blood, the mixture digested at 37–40° C. for half an hour and then centrifuged. The decanted fluid was then tested for its content of immune body. Only when this test proved positive, and it could therefore be assumed that all receptors had been saturated, was the blood so treated employed for injections. But it was previously repeatedly washed with physiological salt solution in order to remove all free immune body. Finally the centrifuged sediment was made up to its original volume. The course of such an experiment is illustrated in the following:

100 cc. ox blood are mixed with 25 cc. inactive immune serum of a rabbit which has been immunized with ox blood. Of this immune serum, 0.0025 cc. suffice, when complement is added, to just completely dissolve 1 cc. 5% ox blood; the amount employed, therefore, represents five times the amount necessary to dissolve the 100 cc. ox blood. After the mixture has remained in the thermostat for half an hour it is filled up to 300 cc. with 0.85% salt solution and centrifuged. The first decantation is tested by adding it in decreasing quantities to 1 cc. 5% ox blood plus 0.4 cc. normal rabbit serum (as complement).

The following results are obtained:

- 1st. Decantation: 1.5 cc. complete hæmolysis.  
                   1.0 cc. almost complete hæmolysis.  
                   0.5 cc.    "        "        "  
                   0.25 strong hæmolysis.  
                   0.1   no        "

From this it must be concluded that the blood-cell receptors are incapable of further absorption; in other words, that they have been saturated.

The second decantation tested in this same manner yields the following result:

- 2d. Decantation: 3.0 cc. strong.  
                   2.0 " moderate.  
                   1.0 " little.  
                   0.5 " trace.

It therefore contains only very little immune body.

The blood is once more washed and centrifuged and then filled up to 100 cc. The blood-cells thus saturated with immune body are injected in rabbits intraperitoneally, each animal receiving 25 cc. of the mixture.

At the same time control animals are injected with the same amounts of normal ox blood.

Usually on the tenth day after the injection, as this had shown itself the most favorable time, serum was withdrawn, inactivated and tested for its content of immune body by adding it in decreasing quantities to 1 cc. 5% ox blood plus sufficient complement. Either rabbit serum, 0.4–0.5 cc., or guinea-pig serum, 0.1–0.15 cc., served as complement, for these are equally well adapted for this purpose. The results of the experiments are as follows:

Out of eight rabbits injected intraperitoneally with ox blood saturated with immune body, *only three corresponded to the requirements which follow from von Dungern's results.* Their serum, tested exactly like the immune body, failed even in amounts of 1.0 cc. to produce a trace of hæmolysis, whereas when the serum of the corresponding control animals was tested, 0.025 and 0.05 cc. respectively sufficed to effect complete hæmolysis.

These results are approached very closely by the serum of a fourth animal. The hæmolytic action of this serum compared to that of the serum of the corresponding control animal was 1: <135, i.e., was exceedingly slight. The remaining four rabbits *had produced an immune body in greater or less amounts*, though this amount was always far less than that produced by the corresponding control animals. When the absence of a zone of marked complete solution rendered it impossible to make an exact determination, the comparison of the immune body values of the sera in parallel tests was accomplished by comparison of tubes whose colors corresponded. The amount of immune body possessed by these animals compared to that of the corresponding control animals was as follows:

(1) 1:5; (2) 1:7; (3) 1:10; (4) 1:10.

I have supplemented these experiments with a smaller series of experiments made with intravenous injections. In these, of course, very much smaller amounts of blood were used for injection because when the blood-cells loaded with immune body are injected directly into the circulation, they suffer hæmolysis through the action of the com-

plement present in the serum, causing serious symptoms or, with larger amounts of blood, fatal results. This accords with the phenomena observed by Rehus<sup>1</sup> when he injected rabbits which had been immunized with ox blood, intravenously with normal ox blood. Only two of the animals I employed, namely those injected with 7-8 cc. blood, remained alive sufficiently long. In one of these only traces of immune body were found in the serum, whereas the serum of the other animal effected complete solution in doses of 0.05 cc. In the serum of a control animal the limit of complete solution was 0.01 cc. These few experiments confirm the results obtained with intraperitoneal injections, *that blood-cells saturated with immune body have not by any means always lost the power to excite a certain degree of immunity reaction in the organism.*

Our results, therefore, show that in half of the animals, in conformity with the results obtained by von Dungern, the power of the blood to cause an immunity reaction is lost, owing to the blocking of that particular group in the blood-cell which unites with the immune body. In the remaining cases, however, the specific immune body was produced, though always in decidedly less amount, since only a fifth to a tenth part of the amount appeared that was produced by the control animals. This apparently unfavorable portion of the experiment shows at least that *saturation with immune body exerts a marked restricting influence.* These results agree with those obtained by Neisser and Lubowski with injections of agglutinated typhoid bacilli.

Furthermore, like Neisser and Lubowski, in an animal which had not reacted to the injection of saturated blood, we found after injection of the same amount of normal blood that an immune body of considerable power had developed in the serum. The complete solvent dose for 1 cc. 5% ox blood amounted to 0.005 cc. serum. These last experiments, which have been done on a much larger scale by Neisser and Lubowski on typhoid bacilli, indicate that the failure of antibodies to form is not due to possible individual differences in the *reacting capacity* of the organism. Considering the uniform appearance of immune body in rabbits treated with ox blood such an assumption would have lacked all probability.

That portion of the experiments in which the injection of saturated blood-cells was borne by the animals without producing any reaction, *can be regarded, as has been done by von Dungern, as a complete demon-*

<sup>1</sup> Rehus, Comp. rend. de la Soc. biol., 1891, No. 12.

stration that the groups exciting the production of immunity are actually the same as those which in haemolysis anchor the immune body. We have seen, however, that there is not always an absence of reaction and that even the injection of the same saturated blood, which in one animal fails to cause a production of immune body, is followed in another animal by a certain low-grade production of immune body. The cause of these phenomena can only be that certain animals possess the individual capacity to anchor the saturated receptors in spite of this saturation. We do not know the mechanism of this action. Two factors in particular come into consideration; a part of the immune body may perhaps be destroyed in the animal body through special agencies (oxidation?) and the receptors thereby set free. It is also possible, however, without assuming a destruction of immune body to explain the phenomenon in the sense of Ehrlich's views, by assuming a higher affinity of the tissue receptors present in the animal body, which receptors then would be able to break up the union of blood-cell receptors and immune body, and draw the blood-cell receptors unto themselves.<sup>1</sup>

Whichever of these explanations is the correct one, our experiments certainly show one thing, that the dissolution of the blood-cell receptor combination is never a complete one. Merely a portion of the groups is concerned, for only by this partial dissolution is the fact (determined by Neisser and Laubowski, as well as by us), to be explained that a very slight degree of immunity reaction is produced by the injection of saturated receptors.

Hence even in the cases running an apparently unfavorable course, only a part always of the receptors exert their action. This portion of the experiments may therefore also be used as a support for the side-chain theory.

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<sup>1</sup> A similar assumption must be made in order to explain certain forms of over-sensitiveness studied particularly by v. Behring, in which, despite a large excess of antitoxin, very small doses of toxin cause death. The most ready explanation is that here, in contrast to the behavior in normal animals, the toxinophile receptors possess a pathologically increased avidity by which they are enabled to break up the neutral toxin-antitoxin mixture (which cannot be broken up by normal cells) and take up the toxin thus set free.

#### XIV. CONCERNING THE ESCAPE OF HÆMOGLOBIN FROM BLOOD CELLS HARDENED WITH CORROSIVE SUBLIMATE.<sup>1</sup>

By HANS SACHS, Assistant at the Institute.

THE following study was undertaken on reading the results of investigations carried on by Matthes<sup>2</sup> on the rôle of the immune body (amboceptor) in hæmolysis. The peculiarity of his very interesting results demands a thorough study of the factors concerned.

The facts there brought out have been confirmed by us, but the results of our study have led us to regard these facts in an entirely different light. As a result of numerous earlier experiences with pepsin, pancreatin and papain we can confirm the observation that normal as well as sensitized red blood-cells (i.e. cells loaded with immune body) cannot be attacked by digestive ferments.<sup>3</sup> With digestive experiments with pepsin and pancreatin, to be sure, the difficulty exists that the amounts of HCl and alkali respectively which represents the optimum of action, are in themselves not indifferent for the blood-cells. With these ferments one is therefore forced to work under relatively unfavorable conditions.

Matthes killed the blood-cells by means of Hayem's solution (which, as is well-known, contains 4% mercuric chloride) and found that *blood-cells so treated were readily dissolved by means of active pancreas fluid*. These fixed blood-cells, which are no longer susceptible to the destructive action even of distilled water, are dissolved by the specific hæmolytic serum and *even by their own normal serum*.

<sup>1</sup> Reprint from the *Muenchener med. Wochenschr.*, 1902, No. 5.

<sup>2</sup> M. Matthes, *Experimenteller Beitrag zur Frage der Hämolyse*, *Muench. med. Wochenschr.*, 1902, No. 1.

<sup>3</sup> According to recent investigations of Dr. Morgenroth, the interesting intestinal ferment, erepsin, described by Cohnheim and by him kindly placed at our disposal, is also not able to attack sensitized blood-cells.

Although we can entirely confirm these statements, we cannot accept Matthes' view, according to which the solution of the fixed blood-cells by pancreatin is conceived as a digestion, the Hayem solution acting somewhat like an immune body. The striking fact that the fixed blood-cells dissolve even in their own serum appeared to us rather to be the result of the union of the mercuric chloride (which adhered to the blood-cells and prevented this solution) with the albumin of the serum. The experiments made in this direction at the suggestion of Prof. Ehrlich have completely confirmed this view.

Following the procedure of Matthes, I employed rabbit blood which, freed from serum, was mixed with Hayem's solution in the proportion of 1:4. After standing a short time, the blood was centrifuged and then washed three or four times with 0.85% salt solution. Finally a 5% suspension of the fixed blood-cells in 0.85% salt solution was prepared. The corresponding control was made with normal 5% rabbit blood.

In the experiments 1 cc. of the 5% blood mixtures was used; the fluid, after the addition of the reagent being made up to 2 cc. with physiological salt solution. It was found that *not only fresh rabbit serum, but even rabbit serum which had been inactivated by half an hour's heating to 56° C., as well as rabbit serum which had been diluted with ten volumes of physiological salt solution and then boiled one hour,* was still able to cause solution of the fixed rabbit blood-cells; 0.075 cc. serum causing complete and almost instantaneous solution. In this case the toxic action of the serum can hardly be thought of. The experiment indicated rather that other kinds of influences are the cause of this curious phenomenon. If the conception is correct that we are dealing with a combination of the mercury with the serum, it should be possible also, with other means which abstract the mercury, to cause a solution of blood-cells fixed with Hayem's solution. As a matter of fact this can very easily be done. I chose potassium iodide and sodium hyposulphite for this test and found that extremely small amounts of these substances cause immediate solution of the fixed blood. 0.00075 cc. of a 20% KI solution in physiological salt solution or 0.00025 cc. of a similar hyposulphite solution sufficed to completely dissolve 1 cc. of our 5% fixed blood suspension.<sup>1</sup> *This positively shows that the function of the serum*

<sup>1</sup> With normal rabbit blood, 1000-2000 times the amount of KI or of hyposulphite solution still acts indifferently.

*albumin in the experiments made by Matthes is that which we assumed.* It must therefore be concluded that the blood-cells treated with Hayem's solution do not dissolve in water because the mercuric chloride with which they have combined prevents the escape of the hemoglobin. The cause of this may be that the soluble substances, e.g. the hemoglobin, form an insoluble combination with the mercuric chloride; it is sufficient, however, to assume that the limiting membrane of the discophasma becomes denser through the deposited mercury salt and so prevents the diffusion of the blood coloring-matter. Be this as it may, certainly all agencies which break up the mercury combination will cause an immediate solution of the hemoglobin. The reason for this is that the discophasma, which in the living state hinders the diffusion of hemoglobin, has been killed by the sublimate treatment.

From this it is easily seen that the solution of the fixed blood by means of pancreatin as it is described by Matthes, is not to be regarded as a species of *digestion*. Every such ferment solution contains enough albumin to explain the action according to our view. I was able to confirm this by the experiment in which the hemolytic action (observed by us also) of *neutral* pepsin and pancreatin solutions was exerted in like manner when the solution had previously been heated to 95° C. for 1 hour.

In conclusion it may be remarked that, after fixation with 4% mercuric chloride solution in physiological salt solution instead of with Hayem's fluid, the blood-cells behaved in exactly similar fashion, as was *a priori* to be expected. The control tests made at the same time with normal blood gave negative results in all the experiments. On the other hand with *solanin*, a substance which dissolves normal blood even in enormous dilutions, hemolysis of fixed blood-cells could not be effected even though large doses were employed. In this substance the necessary albumin is wanting and the dead blood-cells are no longer vulnerable to the action of the blood poison.

To sum up, we may say that in the blood-cells hardened with Hayem's solution it is merely the chemically bound mercuric chloride which hinders the escape of the hemoglobin. All agents which are capable of attracting this salt to themselves, i.e. to "de-harden" the blood-cells, cause the immediate escape of hemoglobin.

Hence, although the observations of Matthes are extremely interesting in themselves, they possess no value for the doctrine of hemolysis. On the other hand it would seem as though they might



be applied to a method of detecting smallest amounts of mercury.

SUBSEQUENT ADDITION.—In a recent communication (Muench. med. Wochenschr. 1902, No. 17) Matthes has completely confirmed the results of our experiments so far as mammalian blood-cells are concerned. The fact that other species of blood, such as frog blood studied by Matthes, after hardening with mercuric chloride, do not give up their haemoglobin even in fluids rich in albumin does not affect our view, but only points to a *high degree of hardening* of the frog-blood stromata which does not permit the escape of the haemoglobin even in the presence of substances abstracting mercury. We did not deny that the stromata could be digested by means of proteolytic ferment. Our objection was directed only to regarding the escape of haemoglobin, an indication of a digestion, or of digesting complements.



## XV. A CONTRIBUTION TO THE STUDY OF THE POISON OF THE COMMON GARDEN SPIDER.<sup>1</sup>

By Dr. HANS SACHS, Assistant at the Institute.

THE studies in hemolysis, constantly keeping pace with the development of the doctrine of immunity, have shown that besides the usual blood poisons sharply defined chemically, there is another group of hemolysins of animal or vegetable origin which exert their damaging influence like the toxins, by combining with certain definite groups of the protoplasm. Included in this are snake venom, numerous bacterial secretions such as tetanolysin and staphylolysin, toxalbumins of higher plants, such as crotin. Besides this there is the endless series of hemolysins, both normal and those produced at will by immunization, which are found in the blood serum.

Of the highest importance for the conception of the similarity of these blood poisons was the fact that *only such blood-cells are sensitive to these hemolysins which are capable of anchoring them*. This fundamental law, which was first recognized and clearly formulated by Ehrlich and Morgenroth<sup>2</sup> has constantly been confirmed, especially in the study of the serum hemolysins artificially produced. *As a result of this the mode of action of these poisons as well as of the toxins has been conceived from the standpoint of the side-chain theory.* " \* \* \* the prerequisite and the cause of the poisonous action in all these cases is the presence in the blood-cells of appropriate receptors (side chains) which fit into the haptophore groups of the toxin; conversely, therefore, there is an intimate connection between natural immunity and the absence of receptors." (Ehrlich.)

It is evident that the study of the combining relations of the toxin-like blood poisons is of great significance for the study of the

<sup>1</sup> Reprint from *Beiträge zur chemischen Physiologie u. Pathologie*, Vol. II, No. 1-3.

<sup>2</sup> See page 1 et seq.

causes of this poisonous action. Such a study, moreover, is calculated to extend our knowledge of the receptors and their physiological distribution in the animal kingdom. While examining extract derived from the common garden spider (*Epeira diademata*) I found in it a hæmolysin which showed itself particularly well adapted to researches in this direction.

The description of a complete experiment will give an idea of the method of obtaining and testing this poison.

A garden spider weighing 1.4 grams is rubbed up with 5 cc. of toluol water containing 10% NaCl and the fluid kept in the refrigerator for twenty-four hours. Then water is added to make the total volume 25 cc. and the mixture filtered (or centrifuged). The hæmolytic experiments are made in the usual manner with this cloudy, brownish-yellow filtrate. Decreasing amounts of the poison solution are placed in a series of test-tubes, each of which is then filled up to 1.0 cc. with physiological (0.85%) salt solution. Each tube then receives one drop of undiluted blood or 1 cc. of a 5% suspension of blood in physiological salt solution. The specimens are kept in the incubator at 37°C. for two hours, and then in the refrigerator until the following day when the amount of solution is determined. The blood employed was always centrifuged and washed in order to remove the adherent serum and so exclude any possible disturbance from that source.

The *Arachnolysin*, as we may designate the active principle of the poison solution, causes solution of the sensitive blood-cells even at room temperature; when present in certain proportions, solution occurs almost instantaneously. In this respect, arachnolysin is somewhat analogous to snake venom, while it differs therein from the hæmolysins of blood serum, in which, as is well known, actual hæmolysis is preceded by a longer or shorter period of incubation. The more exact determinations on different species of blood were made in the usual manner and yielded the results shown in the following table. The amounts of arachnolysin given in the table refer to the original solution, containing 28% of spider substance.

As can be seen from the table we are here dealing with a hæmolysin of extraordinary power, the action of which on the individual species of blood, however, is very variable. Thus a number of species of blood are destroyed even in dilution of 1:1000 or 1:10000 (this refers to the original poison solution); others remain unaffected even by large amounts of poison. Next to rat blood, the most sensitive was rabbit blood, for 0.0001 cc. of the original solution, i.e., 0.000028 g. of spider substance, sufficed to completely dissolve 0.05 cc. of blood (=200,000,000 blood-cells). A garden spider weighing 1.4 g. then

Arachnolysin.		Hæmolytic Action on the Blood of			
		Rabbit.	Rat.	Mouse.	Man.
1/1000	cc. 1.0	complete	complete	complete	complete
	0.75	"	"	"	"
	0.5	"	"	"	"
	0.35	"	"	"	almost complete
	0.25	"	"	almost complete	do.
	0.15	"	"	do.	moderate
1/10000	1.0	"	"	"	"
	0.75	almost complete	almost complete	strong	little
	0.5	strong	strong	"	trace

Arachnolysin.		Hæmolytic Action on the Blood of					
		Ox.	Goose.	Guinea-Pig.	Horse.	Sheep.	Dog.
1/1000	cc. 1.0	complete	strong	0	0	0	0
	0.75	almost complete	"	No hæmolysis even with larger amounts			
	0.5	strong	"				
	0.35	little	"				
	0.25	trace	"				
	0.15	0	"				
1/10000	1.0	—	"				
	0.75	—	moderate				
	0.5	—	"				

fore contains sufficient poison to completely destroy 2.5 liters rabbit blood. Remembering that only an extremely small part of the spider's weight is made up by the active poisonous constituent, and even assuming that the content of arachnolysin amounts to 1%, we see that *this enormous activity indicates that the arachnolysin belongs to the class of blood poisons which exert a powerful action after the manner of the toxins.*

The same is indicated by the marked instability of the active principle. Heat readily destroys the arachnolysin, although a higher degree is necessary than for other hæmolysins. Heating to 56° C. for 40 minutes does not affect the poison solution, and at 60° C. only a very slight reduction of action is noticed. Complete destruction does not occur until the poison is heated to 70°–72° C. for 40 minutes. Arachnolysin is easily preserved by the addition of glycine, showing no reduction in activity even after months.

Experiments, designed to show whether normal sera possess an inhibiting action on hæmolysis due to spider poison, have had nega-

tive results; the sera of man, rabbit, horse, pig, dog, rat, guinea-pig, goat, sheep, ox, goose, and pigeon, inactivated by heating to 56° C. in order to eliminate any possible solvent action, were unable even in amounts of 1.0 cc. to protect rabbit blood against just a complete solvent dose of arachnolysin.

On the other hand, the study of the poison's behavior toward sensitive and insensitive cells has yielded results of special interest in connection with the receptor theory. Certain species of blood, such as dog or guinea-pig blood, have shown themselves immune to the spider poison. This presents the most favorable conditions for studying the relations between the binding of poisons and their action. This point, as we have seen, is of the greatest importance for the view that serum haemolysins are toxin-like bodies.

If arachnolysin is a blood-poison whose action is due to the anchoring of a certain haptophore group to a receptor of the sensitive blood-cell, and if, corresponding to this, the immunity of certain species of blood is due to a lack of appropriate receptors, it follows that the sensitive blood-cells must be able to bind the active principle of such a poison solution, while the insensitive cells leave it entirely unaffected.

So far as the insensitive bloods are concerned, the method of making the experiment is very simple. Dog blood is mixed with a certain quantity of arachnolysin, kept in the incubator for an hour and frequently shaken. Thereupon the blood, which, of course, is unchanged, is separated by means of a centrifuge. The decanted fluid, compared with the original material, shows not the least diminution of its solvent power on rabbit blood-cells. *This shows that the insensitive dog blood is not able to bind the arachnolysin.*

In the case of the sensitive blood-cells, the demonstration of the combining power is much more difficult, for these, when tested in a similar manner are dissolved, so that it is impossible to separate blood-cells and fluid. We can then only operate with the laky blood solution, the inactivity of which permits of no direct conclusion that a binding of the poison by means of receptors had occurred. Furthermore, if the poison solution has lost its power as a result of the action already exerted, there is no means by which this can be determined. It was necessary, therefore, to employ blood-cell material which had been made stable so far as the vital influences of the haemolysis were concerned, without, however, losing its chemical character. For this purpose we used blood-cell stromata, by

which we mean blood-cells deprived of their hæmoglobin by swelling and then again condensing the blood-cell residues. Ehrlich<sup>1</sup> had already (in 1885) pointed out the importance of this true protoplasm of the blood-cells, and had termed it "discoplasma" because of its peculiar character. According to Ehrlich, the main function of this discoplasma is to prevent the escape of the hæmoglobin, and he therefore ascribed the diffusion of the blood coloring-matter to death of the discoplasma. In agreement with this is the fact first described by Bordet<sup>2</sup> and afterward confirmed by Nolf,<sup>3</sup> that it is the stromata which bind the specific serum hæmolysins. We could therefore assume that in our case, in all probability, the arachnolysin would be bound, if bound at all, by the stromata.

In this Institute a method for the production of the stromata, which differs somewhat from the one commonly employed, has proven particularly valuable, especially in studying the receptors. With the usual solution of the blood in distilled water, the separation by centrifuge of the stromata condensed with salt is extremely difficult; and even with suitable species of blood only a small yield is obtained. By previously heating the blood we have found that the subsequent centrifugation is made considerably easier (perhaps because of a kind of coagulation of the blood-cells) and that a plentiful sediment of stromata is thereby assured.

The blood employed is heated on a water-bath at 50°-60° C. for half an hour (depending on the species of blood, ox blood 60° C., rabbit and guinea-pig blood about 54° C.) until, dark brown in color, it just begins to become laky. Thereupon the blood, made up to 6 to 10 volumes by the addition of water and shaken, is mixed with so much salt that this amounts to 1% of the total amount. The mixture is then strongly centrifuged. The stromata remain at the bottom of the vessel in the form of yellowish-white masses, and can be washed by repeatedly adding NaCl solution and centrifuging.

*The stromata so obtained have preserved their receptor property; they bind specific serum hæmolysins, and also, when introduced into the organism, excite the production of specific hæmolytic immune bodies.*<sup>4</sup>

<sup>1</sup> Ehrlich, Zur Physiologie und Pathologie der Blutscheiben, Charité Annalen, X, 1885.

<sup>2</sup> Bordet, Les Serums hémolysiques, etc., Annales de l'Institut. Pasteur, 1900.

<sup>3</sup> Nolf, Le Mecanisme de la globulyse, Annal. de l'Inst. Pasteur, 1900.

<sup>4</sup> It may be recalled that immunization with heated bacteria has been successfully practiced even from the beginning of the study of immunity.

The fact that they have suffered a certain quantitative loss in these properties, owing to the extensive manipulation to which they have been subjected, in no way affects their utility for conducting experiments. In the *qualitative* demonstration of specific immunity the employment of an *excess of receptors* answers all requirements.

In order, furthermore, to meet the objection of a mechanical adsorption of the poison by the stromata, exactly similar controlling experiments were made simultaneously with a blood of the sensitive class, and with one of the insensitive class. As a representative of the former, rabbit blood, which is highly sensitive, was used. For the control, guinea-pig blood, which is not dissolved by arachnolysin, was used. The degree of activity of the poison solution before and after binding was measured by means of rabbit blood.

The stromata sediments derived from each of 40 cc. rabbit blood and guinea-pig blood, are mixed each with 10 cc. of an arachnolysin solution of which 0.025 cc. suffice to just completely dissolve 0.05 cc. rabbit blood. The stromata so treated are digested for half an hour in the water bath at 40° C., being repeatedly shaken. They are then centrifuged. The decanted fluid from the stromata of guinea-pig blood, like the original material, still completely dissolves 0.05 cc. rabbit blood in amounts of 0.025 cc.; the decanted fluid from the rabbit blood stromata, on the other hand, has entirely lost its poisonous action. Even in amounts of 1.0 cc. it is unable to exert the least action on rabbit blood.

Hence the stromata obtained from the sensitive blood have actually bound the arachnolysin, and this combination must be regarded as a chemical one because the control test with guinea-pig blood shows that the insensitive cell-material exerts no attraction whatever on the arachnolysin. Such behavior, however, is most easily explained by assuming, in accordance with the side-chain theory, the presence of appropriate receptors in the sensitive cells as a prerequisite for the action of the arachnolysin. The natural immunity of certain species of blood will then correspond to an absence of appropriate receptors. We see from this that the distribution of receptors capable of binding arachnolysin, at least so far as the blood is concerned, is not universal throughout the animal kingdom, but confined to certain species.

While the experiences already mentioned lead us to regard arachnolysin as a poison belonging to the class of toxins, the evidence will be made absolutely conclusive by demonstrating *the ability of the poison to produce antitoxin*, the most important criterion for the

toxin nature of any substance. Owing to the scarcity of material the immunizing experiments were somewhat delayed; they will, however, be dealt with in detail at the proper time. Nevertheless I can announce that shortly before the conclusion of this work we succeeded, by means of a short immunization of guinea-pigs<sup>1</sup> with garden-spider poison, to produce a high-grade antitoxic serum, of which 0.0025 cc. sufficed to fully protect 0.05 cc. rabbit blood against a complete solvent dose. This proves the toxin nature of arachnolysin.

In conclusion I should like to refer to the relations which arachnolysin bears to what we know about spider poisons in general. In doing so I shall follow Kobert<sup>2</sup> who made the fundamental studies in the toxicology of animal and vegetable poisons, and to whom we owe most of our knowledge concerning spider poisons. In addition to the true secretion of the poison gland, Kobert distinguishes "a toxalbumin which permeates the entire body of the spider, even the legs and eggs, but which bears no necessary relation to the poison gland." In some species of spiders this substance mixes with the gland poison. According to Kobert, the more toxalbumin gets into the wound, the stronger are the constitutional symptoms; the more true gland poison, the stronger the local changes. The latter is especially the case in the lathrodeutes species (*maliniquette*, *karakurte*) whose sting produces most fearful general symptoms, even being able to kill human beings. In these the gland secretion becomes dangerous only when mixed with toxalbumin derived from the body. In contrast to this, the sting of the garden spider produces only local symptoms of irritation, although the spider's body contains a toxalbumin whose action is analogous to the preceding; but this substance does not become mixed with the gland secretion. This being the case, it is very likely that the haemolysin described by us is identical with the toxalbumin already known to Kobert; for we also obtained it from the body substance of the garden spider, and found its properties to be those of the toxin.

**ADDITION ON REVISION.**—Since sending in the manuscript of this study I have learned of a monograph by Kobert (*Beiträge zur Kenntnis der Giftspinnen*, Stuttgart, 1901) which has just appeared. In this Kobert also reports on the haemolytic action of the poison of *Karakurtes* and of garden spiders. He states

<sup>1</sup> Hence although guinea-pig blood is insensitive to arachnolysin, appropriate receptors capable of binding the poison must be present in the guinea-pig organism outside of the blood.

<sup>2</sup> Kobert, *Lehrbuch der Intoxicationen*, Stuttgart, 1893, p. 329



that although he found the hemolytic action to be present in the latter, "it was much less than that of Karakurtes poison." It is possible, however, that Robert made these experiments on one of the species of blood found by us to be insensitive to arachnolyon (horse blood, dog blood). At any rate our garden-spider extract far exceeds in hemolytic action the Karakurtes poison tested by Robert in this respect. I should also like to point out that for the hemolytic experiments with Karakurtes poison, Robert used dog blood, which according to our table belongs to the class of blood species immune to garden-spider poison. Perhaps in conformity with the extensive analogy between these two spider poisons, the Karakurtes poison possesses a far greater hemolytic action on other species of blood. Robert's observation that a tolerance can be established against Karakurtes poison as well as against garden spider poison, agrees very well with the idea of a strong antitoxic serum, a fact actually observed by us. Since then we have obtained such a serum also in rabbits.



## XVI. A STUDY OF TOAD POISON.<sup>1</sup>

By Dr. FR. POESCHKE.

THE numerous investigations concerning toad poison which have been made especially by French and Italian workers, have not yet come to a definite conclusion as to whether this substance is alkaloid-like or toxin-like. The skin secretions of the different varieties of toads contain a number of bodies which have not thus far been studied. In the garlic toad, for example, there is a substance of garlicky odor, which has not been more closely identified. Besides this, according to Calmels, toad secretion contains methyleurbylaminic acid and methyleurbylamin, which are said to act intensely on the nervous system. Kobert applied the name "phrynin" to a substance which irritates the mucous membranes very intensely. Phasalis and Bertrand claim to have isolated an alkaloid from the blood serum of the common toad, but it remains doubtful whether the substance was not a toxin, for they were unable to produce it in chemically pure form. At the conclusion of their investigations they themselves say that the poisonous action is not due entirely to the "alkaloid." In like manner Jornara and Casali claim to have isolated "bufidin" from dried toad poison. They say that this forms crystalline salts and must therefore be an alkaloid. The alcoholic extract of toad skin is said to have an action similar to digitalis. Pugliese found that toad poison changes haemoglobin into methaemoglobin, and that it also dissolves the blood-cells outside the body. Pugliese has not attempted any more detailed investigation. From the abstracts of his study at my disposal I was unable to determine the species of toad used in his experiments.

The object of the following investigation is to furnish a small contribution to our knowledge of toad poison. At present there can be no thought of any exact analysis of the poison.

<sup>1</sup> Reprint from *Beiträge zur chemischen Physiologie u. Pathologie*, Vol. 1, Nos. 10-12.

## METHOD OF OBTAINING THE POISON.

The toad poison used in my experiments was derived from *bombinator igneus*, the fire toad, and from *bufo canorus*, the common garden toad. In order to obtain the poison, the skin of the abdomen and back of a freshly captured toad was used, for the poison is present in largest amounts in the skin. The muscles and blood serum of the fire toad also contain the poison, but in smaller quantities.

After the toads were thoroughly rinsed with physiological salt solution they were decapitated and skinned. The skin was again rinsed with salt solution and then rubbed to a paste, as homogeneous as possible, with powdered glass. After adding 2 to 3 cc. physiological salt solution the mixture was filtered or centrifuged. The resulting fluid had a feebly acid reaction, a greyish white color and a peculiar, garlicky odor. Toluol was added as a preservative, and the fluid stored in the refrigerator. In the same manner I prepared an extract from the skin of the garden toad.

The extract of the skin of the fire-toad showed strong hemolytic properties; that of the garden toad the same, though only in traces. (See Table III.) The following experiments refer only to the fire-toad poison which, for short, we shall call "phrynolysin." The poison of the garden toad was used merely for comparison.

## PROPERTIES OF PHRYNOLYSIN.

Phrynolysin is an exceedingly labile body. Heating to 56° C., exposure to light, the addition of alcohol, ether, chloroform, mineral acids, strong potash lye, pepsin and trypsin, all destroy it in a short time. Drying the phrynolysin over anhydrous phosphoric acid at room temperature weakens it materially. It does not dialyze.

Since, as already mentioned, the extract from the toad skin possesses a faint acid reaction, requiring 1 to 1.3 cc. decinormal lye for neutralization, it could be assumed that the acid reaction slowly destroys the toxin. The destruction of the toxin, however, proceeds in the same time in neutral as in feebly acid solution, so that the acid reaction cannot possess any great influence. The hemolytic action is the same in acid as in neutral solution.

The best preservative for this substance is toluol, first employed by Ehrlich for preserving the toxins. Cold storage is also good. After a time the fluid becomes cloudy, owing to the separation of albumin, but it maintains its hemolytic power unimpaired for a

considerable time. After from one to two months the phrynolysin gradually becomes inert. Owing to the extreme lability of the toxin there can, for the present, be no thought of obtaining the substance pure, for even drying at room temperature weakens the poison considerably. Owing to lack of material, a pharmacological examination of the poison could not be undertaken.

#### BEHAVIOR OF THE PHRYNOLYSIN TOWARD DIFFERENT SPECIES OF BLOOD.

The method of testing was such that a series of test-tubes was prepared, each containing 1 cc. of the dilution 1:10, 1:20, etc., i.e., decreasing amounts of the poison. The dilutions were made with 0.85% salt solution. To each tube 1 cc. of the 5% blood suspension in 0.85% salt solution was added. Thereupon the tubes were kept at 37° C. for two hours and in the refrigerator overnight. A "complete solution" is one that on shaking shows no body elements of any kind: "almost complete" if there is still a slight sediment, and "incomplete" when numerous blood-cells are undissolved. This is followed in order by "red," "top," "trace," "0."

Commencing with Table III all the experiments are made on sheep blood.

As can be seen from Table I, sheep blood is most strongly dissolved, frog and toad blood not at all. The limits of solution for sheep blood are a dilution of 1:10240 in the case of phrynolysins I and II, and 1:5120 in phrynolysin III. In Table IV, decreasing amounts of the poison are added to 1 cc. 5% sheep blood. Of phrynolysin I, 0.0025 cc. sufficed to effect complete solution; of II and III, 0.00025 cc. sufficed, and of IV, 0.005 cc. By determining the amount of dry residue in poison solution II it is seen that 0.0000022 g. of organic substance suffice to completely dissolve 1 cc. 5% sheep blood. Of poison solution III, 0.0000015 g. have the same effect. If we assume that one-tenth of this organic substance (probably it is still less) represents true phrynolysin, the rest being merely indifferent albuminous bodies, we find that  $\frac{2}{10}$  mg. are sufficient to completely dissolve one liter of sheep blood.

The yield of phrynolysin is subject to individual fluctuations. Animals freshly caught yield a stronger haemolysin than those which have been kept in captivity for some time.

TABLE I.

Dilution.	Sheep Blood.	Goat Blood.	Rabbit Blood.	Dog Blood.	Ox Blood.
1:20	complete	complete	complete	complete	top
1:40	"	"	"	"	"
1:80	"	"	"	red	"
1:160	"	"	"	top	trace
1:320	"	"	red	"	0
1:640	"	"	"	"	0
1:1280	"	"	"	"	0
1:2560	"	incomplete	trace	"	0
1:5120	almost complete	top	0	0	0
1:10240	red	trace	0	0	0
1:20480	trace	0	0	0	0
1:40960	0	0	0	0	0

Dilution.	Chicken Blood.	Guinea-pig Blood.	Rat Blood.	Pigeon Blood.
1:20	incomplete	red	red	trace
1:40	red	"	top	0
1:80	"	top	"	0
1:160	0	trace	trace	0
1:320	0	0	0	0

Dilution.	Pigeon Blood.	Goose Blood.	Frog Blood.	Toad Blood.
1:20	trace	red	0	0
1:40	0	top	0	0
1:80	0	0	0	0

TABLE II.

PHRYNOLYSIN OF THE COMMON GARDEN TOAD.

Dilution.	Sheep Blood.	Goat Blood.	Dog Blood.	Rabbit Blood.	Guinea-pig Blood.	Ox Blood.
1:20	red	0	red	0	0	0
1:40	trace	0	top	0	0	0
1:80	"	0	"	0	0	0

TABLE III.

BEHAVIOR OF DIFFERENT PHRYNOLYSINS TOWARD SHEEP BLOOD.

Dilution.	Phrynolysin I.	Phrynolysin II.	Phrynolysin III.
1:640	complete	complete	complete
1:1280	"	"	"
1:2560	"	"	"
1:5120	"	"	almost complete
1:10240	almost complete	almost complete	top
1:20480	top	red	red

TABLE IV.  
BEHAVIOR OF DIFFERENT PHRYNOLYSINS TOWARD SHEEP BLOOD

cc.	Phrynolysin I.	Phrynolysin II.	Phrynolysin III.	Phrynolysin IV.
0.005	complete	complete	complete	complete
0.0025	"	"	"	incomplete
0.001	incomplete	"	"	top
0.00075	red	"	"	0
0.0005	top	"	"	0
0.00025	0	"	"	0
0.0001	0	incomplete	red	0

ATTEMPTS AT REACTIVATING A PHRYNOLYSIN WHICH HAD BECOME  
INACTIVE AT 56° C.

The investigations of Ehrlich and Morgenroth have shown that the hemolysins of the higher vertebrates are of complex constitution. They consist of two portions, the complement and the immune body. By heating to 56° C. the complement is destroyed, while the immune body remains intact. The immune body by itself can not exert any hemolytic action; a fitting complement must first be added.

It would be quite comprehensible for the phrynolysin likewise to consist of two parts. Heating to 56° C. would destroy the complement, while the thermostable interbody would be preserved. I therefore attempted to reactivate the toxin which had become inactive at 56° C. and tried the addition of a number of different normal serum for this purpose, such as goat serum, sheep serum, pigeon serum, horse serum, guinea-pig serum, and rabbit serum, all without success, no solution taking place. Unfortunately, owing to lack of material, I was unable to obtain 1 or 2 cc. of serum from the fire-toad in order to employ this for reactivation. Experiments with the normal sera of the higher vertebrates are not conclusive, because the complement sought for may possibly be contained only in the serum of the fire-toad. For the present therefore the question as to the complex character of the phrynolysin must still be kept open.

DO NORMAL SERA CONTAIN ANTIBODIES AGAINST PHRYNOLYSIN?

A number of normal sera, which had first been inactivated at 56° C. in order to avoid solution of the sheep blood added, were tested for this purpose, e.g., pigeon serum, sheep serum, guinea-pig serum, horse serum, rabbit serum, and goat serum. None of these sera,

even in amounts up to 1 or 2 cc. was able to prevent solution, although only the single solvent dose of phrynolysin was added to the mixture of blood and serum.

#### IMMUNIZATION WITH PHRYNOLYSIN.

In order to furnish conclusive proof that phrynolysin is a true toxin, a number of rabbits were immunized with the same. The poison was injected subcutaneously, commencing with  $\frac{1}{2}$  cc. and increasing to 5 cc. in the course of eight days. The dose of 5 cc. was then injected every 5 to 6 days for two or three times so that in the course of three weeks about 30-35 cc. had been given.

It is not advisable to give more than 5 cc. at once because otherwise the animals die in one or two days. The anatomical findings in an animal which has died of toxic poison are negative, excepting a marked hyperemia of the abdominal viscera; no macroscopical changes of the organs are demonstrable.

As already mentioned, normal rabbit serum does not contain a trace of anti-body against phrynolysin. The production of anti-toxin commences about fourteen days after the injection of the toxin and reaches its maximum in three weeks. Of the strongest serum which I obtained, 0.025 cc. protected against double the solvent dose of phrynolysin for 1 cc. 5% sheep blood.

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## XVII. CONCERNING ALEXIN ACTION<sup>1</sup>

By Dr. HANS SACHS, Assistant at the Institute.

AFTER the fundamental studies of Bordet, and of Ehrlich & Morgenroth had shown that the haemolysins produced in serum by immunization with blood-cells owed their effect to the combined action of two substances (antibody and complement) it seemed very natural to suppose that the haemolysins of normal sera, which had been known for some time, were also of a complex nature. Buchner, who was the first to recognize the significance of the bactericidal and globulicidal properties of blood serum, had conceived these actions from a unitarian standpoint and referred them to the "alexin" of each particular serum. Recent investigations, however, have shown that Buchner's alexin is not a single simple substance, but the sum of an infinite number of combinations, whose more thorough analysis has been rendered possible only by the methods of the newer haemolysin investigations.

The credit of applying the experiences gained with the haemolysins artificially produced to the study of haemolysins of normal serum, belongs to Ehrlich and Morgenroth. They made use of a method which they had already employed in the analysis of haemolytic immune sera, the separation by means of cold. This depends on the fact that at 0° under favorable circumstances only the antibody, and not the complement, is bound by the blood-cells. Accordingly by appropriate treatment it could be shown that the serum had lost part of its power, but that it could be regenerated by the addition of the same kind of serum previously inactivated by heat. This confirmed their view of the complex nature of normal haemolysins. They were further able to activate inactive haemolytic normal sera by the addition of other kinds of sera which served as complements, and which by themselves did not dissolve the particular blood cells

<sup>1</sup> Reprint from the Berl. klin. Wochens. 1902, Nos. 9 and 10



used. This showed conclusively that *the globulicidal property of normal serum is due to the co-action of two bodies, one which withstands heating (thermostable) and the other which does not (thermolabile).*

These views have been accepted by the majority of investigators, and numerous observers, P. Müller,<sup>1</sup> London,<sup>2</sup> E. Neisser and Döring<sup>3</sup> have constantly added new facts, the analysis of which in every instance demonstrates the complex nature of normal hæmolysins. Nevertheless it does not seem to me superfluous to thoroughly discuss this question once more, since such eminent authorities as Buchner<sup>4</sup> and Gruber,<sup>5</sup> because of the negative result of part of their experiments, hold that Ehrlich and Morgenroth's conception of the nature of normal hæmolysins is erroneous. Ehrlich and Morgenroth had from the beginning stated that the solution of this problem in any particular case was only possible by their method *under certain favorable circumstances*. Now, although Buchner and Gruber have employed this method, so that a negative result proves nothing whatever, in consideration of the importance of the matter I have followed the suggestion of Prof. Ehrlich,<sup>6</sup> and undertaken a critical study of the negative findings of these authors. The results of this have already been briefly alluded to elsewhere.

Buchner sought to discover the presence of thermostabile bodies (his "Hilfskörper") on the occurrence of hæmolysis, by reactivating normal sera, which had been inactivated by heating to 60° C., with fresh serum of a different species. But out of the large number of possible combinations he chose only one and used as a source of complement only that serum which was derived from the same species that furnished the blood-cells. In an address on the protective bodies of the blood, delivered at the Hamburg Congress of Naturalists, Ehrlich pointed out that this procedure was inapplicable. It can surely not be expected that every serum contains a fitting

<sup>1</sup> P. Müller, Über Antihæmolysine, Centralblatt für Bacteriologie, Vol. 29, 1901.

<sup>2</sup> E. S. London, Contribution à l'étude des hémolysines, Arch. des Sciences biolog. (Inst. impérial de méd. exper. à St. Petersburg), T. VIII, 1901.

<sup>3</sup> E. Neisser u. Döring, Zur Kenntniss der hæmolytischen Eigenschaften des menschlichen Serums, Berl. klin. Wochen. 1901, No. 22.

<sup>4</sup> Buchner, Sind die Alexine einfache oder complexe Körper? Berl. klin. Wochenschr. 1901, No. 33.

<sup>5</sup> M. Gruber, Zur Theorie der Antikörper, II, Über Bacteriolyse u. Hæmolyse, Münch. med. Wochenschr. 1901, 48 and 49.

<sup>6</sup> Ehrlich, Vortrag im Verein für innere Medicin, Dec. 16, 1901.



complement for any given amboceptor. In testing a series of combinations, therefore, the finding of a suitable complement will to a certain extent be merely a coincidence. In all the cases studied at this Institute, however, even though often only after considerable labors, this has always led to a certain realization of the complex nature of the haemolysin.

Buchner was successful in two of his cases in activating the combination chosen by him: *blood-cells A + inactive serum (amboceptor) B + active serum (complement) A*. (Guinea-pig blood and ox serum; goat blood and rabbit serum.) In three other cases, however, he was unable with a corresponding mode of procedure to restore the solvent power which had been lost by inactivation. Guinea-pig blood and sheep serum (Case I); sheep blood and rabbit serum (Case II); guinea-pig blood and dog serum (Case III). These results, to be sure, are contrary to those of Ehrlich and Morgenroth, who observed more or less marked haemolysis in these same combinations. These opposing results are, however, explained first by the fact that the amount of complement contained in the serum of the same species is subject to individual and chronological variations within wide limits. Beside this, recent experiences, which we shall subsequently discuss in detail, have shown us that the temperature at which the serum is inactivated is not indifferent for the function of the amboceptor. Hence it appears significant that in these experiments Buchner inactivated the sera by heating to 60° C., whereas ordinarily this is done at 56°-57° C. As a matter of fact, Buchner's experiment No. 6 shows that dog serum, in this experiment inactivated by heating only to 57° C., is activated in its haemolytic action for guinea-pig blood by rabbit serum. In view of this, the negative findings of Buchner in Case III lose their significance.

In the three cases looked upon by Buchner as negative, I tried, by separation by means of cold, to convince myself of the presence of two substances effecting the haemolysis. My method of procedure was as follows:

Two parallel series of tubes of blood containing decreasing amounts of active serum were prepared, kept at 0° C. for 2-3 hours and then centrifuged. The decanted fluid of one series was then allowed to act on the sediments of native blood, that of the other series on the sediments of blood which had been treated with a like quantity of inactivated serum. The amount of blood, as in all our experiments, was 1 cc. of a 5% suspension in .85% salt solution.

In two combinations (Cases I and II) the separation of the two components was effected without any trouble. The following protocol will also show the technique of the experiment.

Negative case I of Buchner. 0.5 cc. sheep serum is still just able to completely dissolve guinea-pig blood. To each 1 cc. of a 5% guinea-pig blood suspension varying amounts of active sheep serum are added and the volume of fluid made up to 2 cc. with physiological salt solution. Two parallel series like this are kept at 0° C. for two hours and then centrifuged. The clear decanted fluids from the one series are allowed to act each on the sediment of 1 cc. native 5% guinea-pig blood; the fluids from the other series, each on the sediment of 1 cc. 5% guinea-pig blood, which had previously been treated with the same varying amounts of inactive sheep serum. The hemolytic action of the decanted fluids is shown by Table I.

TABLE I.  
ABSORPTION OF SHEEP SERUM BY GUINEA-PIG BLOOD AT 0° C.

Amount of the Sheep Serum Added  cc.	Solvent Power of the Decanted Fluids for	
	A. Native Guinea-pig Blood.	B. Guinea-pig Blood Previously Treated with Inactive Sheep Serum
1 0.7	moderate	complete
2 0.6	"	"
3 0.5	little	"
4 0.4	trace	almost complete
5 0.35	"	strong
6 0	0	0

Buchner's second negative case deals with the combination sheep blood and rabbit serum. In the following experiment, entirely analogous to the preceding, the complete solvent dose of rabbit serum for sheep blood was 0.2 cc. See Table II.

These experiments, which are confirmed by numerous parallel experiments, show that *in these two cases, as a matter of fact, hemolysis depends on the presence of two substances.* One of these, thermostable, is bound by the blood-cells at 0° C., the other, thermolabile, is left behind at this temperature. The latter, however, is only then able to effect hemolysis when it acts on blood-cells which have previously anchored the thermostable substance, the amboceptor.

A comparison of Tables I and II also shows how much the combining relations between amboceptor and blood-cell on the one

hand, and amboceptor and complement, on the other, may vary from case to case. Whereas in Case II the decanted fluids were

TABLE II.

ABSORPTION OF THE RABBIT SERUM BY SHEEP BLOOD AT 0° C.

Amount of Rabbit Serum Added.  cc.	Solvent Power of the Decanted Fluids for	
	A, Native Sheep Blood.	B. Sheep Blood Previously Treated with Inactive Rabbit Serum.
1 0.6	trace	complete
2 0.45	0	"
3 0.35	0	almost complete
4 0.25	0	moderate
5 0.2	0	"
6 0	0	0

absolutely inactive against native blood (i.e., all the amboceptor had been bound at 0° C. by the blood-cells) in Case I the decanted fluids were then still active when the amounts of serum added were less than the solvent dose. This indicates that in this case the affinity of the amboceptor's cytophile group for the receptor of the cell is relatively slight at 0° C. In like manner the columns B of the tables show a certain difference of affinity between amboceptor and complement. In Case I the decanted fluid still contains the entire complement; in Case II, on the other hand, a portion of the complement must have combined with the amboceptor, for the decanted fluid shows a distinct loss of complement. The separate examination of the sediments of the specimens to which active serum was added agrees with this; in Case I these sediments mixed with physiological salt solution and placed into the incubator showed no trace of solution, while in Case II the sediments of the first three tubes showed moderate, little, and trace of solution respectively.

*Both normal hæmolysins (Buchner's negative Cases I and II) therefore correspond in their main behavior. They consist of two components (readily separable by the "cold method") which in their mutual relations manifest a certain variation in the behavior of their receptors.*

The conditions in these two combinations were favorable for analysis of the mode of action by means of our method. In the study of Buchner's third negative case, however (guinea-pig blood and dog serum), difficulties presented themselves which at first ap-

peared to be insurmountable. Despite numerous variations in the conditions of the experiment we did not succeed with appropriate procedures in effecting a separation by means of the "cold method." The fluids decanted from the mixture of guinea-pig blood-cells and active dog serum manifested the same behavior, so far as hemolytic action was concerned, on normal guinea-pig blood and such as had previously been treated with inactive dog blood; and yet they showed slight differences so that we did not feel justified in drawing any conclusion. However, we soon became convinced that a separation of two substances causing hemolysis had nevertheless been effected by the absorption in the cold. We allowed the fluid decanted from the guinea-pig blood-cells previously treated with active dog serum, which fluid only slightly dissolved native guinea-pig blood, to act on guinea-pig blood sediments, which also had previously been mixed with active dog serum. We were then able to determine that these sediments were strongly, in appropriate quantities completely, dissolved by the decanted fluid, although when mixed merely with salt solution and placed into an incubator they did not dissolve at all, or dissolved only in traces. An experiment of this kind is shown in Table III.

TABLE III.  
ABSORPTION OF DOG SERUM BY GUINEA-PIG BLOOD AT 0° C.

Amount of Dog Serum Added	Hemolysis of the Sediments here poured in Salt Solution at 37°	Solvent Power of the Decanted Fluids for		
		A. Native Guinea- pig Blood	B. Guinea-pig Blood Previously Treated with Inactive Dog Serum	C. Guinea-pig Blood Previously Treated with Active Dog Serum at 0°
1 0.25	trace	almost complete	complete	complete
2 0.2	faint trace	strong	almost complete	"
3 0.15	0	moderate	moderate	"
4 0.1	0	little	little	"
5 0.075	0	trace	trace	strong

Hence by means of the absorption with guinea-pig blood in the cold, the active dog serum was separated into two components each of which by itself was incapable of effecting solution. One of these became attached to the red blood-cells, the other remained in the fluid. The former therefore corresponded in its behavior to the amboceptor, and it was only a coincidence that dog serum inactivated by heating to 60° C. was unable also to assume that rôle. We hoped to discover more about the nature of this curious behavior by employing a different method of inactivat-

ing the dog serum, and therefore in this case turned first to the completion method. By means of completion of the variously inactivated dog serum with other sera which do not dissolve guinea-pig blood, we hoped to obtain an insight into the circumstances here presented. In this way we were able to convince ourselves that dog serum which had been inactivated by half an hour's heating to 60° C. according to Buchner's procedure, is no longer activated in its hæmolytic action on guinea-pig blood, by the addition of guinea-pig serum. When the dog serum, however, was heated only to 55° C. or even only to 50° C. it was always possible to activate such an inactivated serum by means of guinea-pig serum. This was the more readily effected, the lower the inactivating temperature employed. It need hardly be mentioned that in particular cases we always determined whether the serum really was inactive; and this showed that dog serum loses its hæmolytic property for guinea-pig blood completely, even when merely warmed for half an hour to 49° C. We must therefore regard it as a fortunate coincidence that the complement of dog serum is so *markedly thermolabile*, for only under this condition could it be possible to preserve the amboceptor intact, i.e., capable of reacting, for that body is but little more stable. Whether the amboceptor heated to 60° has been damaged in its cytophile or complementophile affinity is still undetermined. One could perhaps also think of a blocking of the complementophile group of the amboceptor due to a binding of the complement taking place at the higher temperature. Be this as it may, these experiments certainly show that the *power of a dog serum (inactivated at a suitable temperature, e.g., 50° C.) to be activated by guinea-pig serum is lessened by heating the dog serum to 55° C. and destroyed at 60° C.*

Table IV shows such an experiment:

TABLE IV.  
COMPLETION (WITH GUINEA-PIG SERUM) OF DOG SERUM INACTIVATED AT  
DIFFERENT TEMPERATURES.

Amount of the Activated Guinea- pig Serum.		Degree of Solution of the Guinea-pig Blood Mixed with 0.15 cc. Dog Serum and Inactivated by Half an Hour's Heating to		
		A, 60°.	B, 55°.	C, 50°.
1	0.5	0	moderate little trace 0	complete strong little 0
2	0.25			
3	0.1			
4	0			

We now repeated the experiment of separation in the cold by allowing the fluid which was decanted from the guinea-pig blood-cells after these had been treated with active dog serum at 0° C., to act on guinea-pig blood sediments previously mixed with dog serum. Our results were in accord with the above and led to a clear understanding of our previous negative findings. See Table V.

TABLE V.  
ABSORPTION OF DOG SERUM BY GUINEA-PIG BLOOD AT 0° C.  
(0.075 cc. dog serum just completely dissolves 1 cc. 5% guinea-pig blood)

Amount of Dog Serum Added,  cc.	Solvent Power of the Decanted Fluids on			
	A. Native Guinea-pig Blood.	B. Guinea-pig Blood Previously Treated with Dog Serum Inactivated at		
		I. 00°.	II. 55°.	III. 50°.
1 0.15	complete	complete	complete	complete
2 0.1	moderate	almost complete	"	"
3 0.075	little	moderate	strong	"

In this case, therefore, we have demonstrated a thermolability of the amboreceptor<sup>1</sup> which shows itself especially in the activating experiment with guinea-pig complement, but also in that with its own dog (complement). Only through this thorough analysis was it possible to furnish for Buchner's third negative case also positive proof of the complex constitution of normal haemolysins.

After having determined that certain amboreceptors will only

<sup>1</sup> It is therefore not at all permissible to define the two components of the haemolysin, as Gruber would do (*Discussion of Gruber's Address*, Wiener Klin. Wochensh. 1901, No. 50), only according to the temperature, and to say that at a certain degree of heat the amboreceptor remains intact while the complement does not. As long ago as their second communication Ehrlich and Morgenroth described a thermostable complement of goat serum which remained intact at 55° C.; and according to our experiences here described a general definition of amboreceptors as bodies which withstand heating to 55° C. is absolutely impossible. The influence of temperature on amboreceptor and complement varies from case to case. Hence that these two factors act together in haemolysis we know only from this, that two substances, in themselves not capable of causing solution, when combined, effect haemolysis; and that one of these substances (the complement) can never alone be bound by the blood-cells but always only through the intervention of the other (the amboreceptor).

bear slight warming, in order to remain capable of reacting, we had to abandon our custom of inactivating sera by simply heating them to 60° C. Thereafter we had always first to determine the minimal inactivating temperature for each individual case. The limits of temperature can usually be determined accurately; for dog serum it is 49° C. We have also tried by means of other complements to activate dog serum inactivated at 50°, and have found a suitable complement not only in guinea-pig serum but also in human serum. In this case also, the thermolability of the amboceptor showed itself, for heating to 60° C. destroyed the reactivability. In two cases, however, the power to reactivate was preserved to a greater or less extent even after heating to 60° C. In like manner dog serum could be activated by the complements described when it had been deprived of its solvent power by other means. Thus the complements of dog serum were absorbed by means of yeast, and by means of an anticomplement serum (from a goat) whose normal amboceptor for guinea-pig blood had been removed by washing with guinea-pig blood. The dog sera so inactivated manifested their amboceptor properties when they were appropriately activated.

In the first two negative cases of Buchner, separation in the cold had shown the presence of amboceptors in the sheep and rabbit serum. I now sought by means of activating experiments to find fitting complements for these amboceptors in other sera. Naturally, after the above experiences, it was necessary here also to first determine the minimal inactivating temperature. For sheep serum this is 50° C., for rabbit serum 51° C. If sheep serum is inactivated by half an hour's heating to 50° C., it is easy to restore the hæmolytic action on guinea-pig blood (Buchner's Case I) by the addition of fresh human serum. In this way the complex nature of the normal hæmolysin of sheep serum can be demonstrated. One can also activate with guinea-pig serum, although then a feebler solvent action is obtained. In both cases the thermolability of the amboceptor is readily demonstrated; for by heating the sheep serum to 60° C. this can no longer be activated, or only in very much less degree.<sup>1</sup>

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<sup>1</sup> In addition to this I have also demonstrated a thermolability of the amboceptors of goat serum which are activated by horse serum and act on rabbit and guinea-pig blood. Repeated investigations by Dr. Morgenroth have shown that a markedly thermolabile amboceptor is contained also in horse serum. This amboceptor, which fits guinea-pig blood, and can no longer be



The combination, sheep blood and rabbit serum (Buchner's second case) presents entirely analogous conditions. Both guinea-pig serum and human serum, the latter only in a moderate degree contain a complement which activates the amboceptor of rabbit serum. The rabbit amboceptor, however, is evidently of more stable constitution; for even after heating to 60°, its solvent power can be completely restored. I can therefore confirm the facts found by Buchner in this case, namely that sheep serum is incapable of restoring the solvent power for sheep blood. This, however, according to the above statement, is naturally no argument against the complex nature of the hæmolyisin *because not every serum need contain a fitting complement for every particular amboceptor.*

Provided that sufficiently numerous combinations are examined, the "completion method" as a rule leads to the positive demonstration of the amboceptors. The "separation in the cold" on the contrary, owing to the peculiarity of the combining relations of the separate components, is entirely inapplicable in a number of cases.

Gruber, the second author to come out against the conception of the complex nature of normal serum hæmolyisins, sought to demonstrate amboceptors in a number of normal sera, by means of "separation in the cold." In view of the preceding it is not surprising that he failed in a number of cases to effect a separation of the hæmolyisin.

Ehrlich and Morgenroth in their second communication on hæmolyisins have already analyzed the conditions for separating the interbody by means of absorption, emphasizing "*that the solution of the problem therefore is now possible only under either of the two above mentioned favorable conditions; (1) When the two haptophore groups of the interbody differ greatly in their affinity; and (2) when, by means of a combination whose discovery depends on chance, an activating complement is found.*"

The limitations of the two methods applicable to an analysis of the complex nature of hæmolyisins, are therefore sharply defined. In any individual case when one method fails, it will always be necessary to make use of the other in order to gain an insight into the constitution of the hæmolyisins at all commensurate with the means at our disposal. The schematic application of only one

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activated after heating to 55° C. can be shown to exist in *active* horse serum (which does not dissolve guinea-pig blood) by combining and completing it with guinea-pig serum.



method can lead to the greatest errors. In this respect a comparison of the results obtained by Buchner and Gruber, is very instructive, for among their cases are two combinations which are designated by the one as positive, and by the other negative.

The amboceptor of rabbit serum for sheep blood, which Buchner, because of the failure to reactivate this with sheep serum, regarded as absent, Gruber, by means of the cold separation method, could demonstrate as present; and for ox serum, whose amboceptor Buchner had already demonstrated by the activation with guinea-pig serum, Gruber, through the failure of his cold absorption method, arrived at the view of a pure alexin action.

In Gruber's negative cases, which embrace the following combinations: I, rabbit blood—dog serum; II, rabbit blood—ox serum; III, guinea-pig blood—ox serum, IV, rabbit blood—guinea-pig serum; I have systematically sought for sources of fitting complements and have found these in abundance. Naturally in view of the experiences above mentioned the inactivation of the sera was effected at the lowest temperatures possible; thus dog serum and guinea-pig serum at 50°, ox serum at 52° C. In the following sera (in part in agreement with other previous experiences) I have found complements suitable for activation:

I. *For the amboceptor of dog serum, acting on rabbit blood; in guinea-pig serum, ox serum, goat serum, and sheep serum.*

II. *For the amboceptor of ox serum, acting on rabbit blood; in guinea-pig serum, rabbit serum, and rat serum.*

III. *For the amboceptor of ox serum, acting on guinea-pig blood;—in guinea-pig serum, human serum, rat serum, horse serum, and to a slight extent also in sheep serum.*

Naturally in all the experiments, control tests were made with the active serum, which served as complement. In the cases designated as positive completion, this serum by itself had to exert no hæmolytic action or at least to act in a very much smaller degree.

Gruber's fourth negative case, rabbit blood and guinea-pig serum, offered considerable difficulties because the combination is very little or not at all effective, and it is probably because of this that Gruber speaks of "concentrated guinea-pig serum." Among a large number of guinea-pig sera examined for this purpose, we found only two sufficiently hæmolytically active. But here also, through the successful activation by means of human and ox sera (sera, to be sure, which by themselves dissolve rabbit blood, but which still

effect complete hæmolysis as complements in amounts in which alone they are entirely inert) we could furnish positive proof of the presence of amboceptors.

Buchner and Gruber have therefore described a total of seven cases said to show pure alexin action; and these cases were held by them to be sufficient to decide in the negative the entire question of the complex nature of the normal serum hæmolysins. *Against this we have in all these cases brought positive proof that the "alexin," conceived by Buchner to be a simple unit, always produces its effects through the co-action of two components, the existence of which is demonstrable in different ways. We must therefore uphold Ehrlich and Morgenroth's view, that normal and artificially produced hæmolysins exert their action according to exactly the same mechanism.*

We do not yet possess a method *generally applicable* to demonstrate the complex nature of the hæmolysin, and even a thorough analysis, therefore, need not necessarily achieve the desired result in every case. The method adopted by Muller<sup>1</sup> for demonstrating the amboceptors in chicken serum, which is hæmolytic for rabbit blood, is of interest in this connection. When the usual methods failed he found that bouillon injections caused an increase in the amount of complement in the chicken serum without affecting the amount of amboceptor. This led him to recognize the complex nature of the hæmolysin, a fact confirmed by the successful activation of heated chicken serum by means of pigeon serum. When therefore in isolated cases the separation does not succeed according to the methods heretofore employed, such results, the product of incomplete methods, most certainly do not argue for a simple alexin action. We hope that the employment of the lowest possible temperatures in inactivation will result in increasing "completion" possibilities and make the demonstration of the complex constitution of the hæmolysins easier in difficult cases. At present this demonstration has failed only in the case of eel serum (which, to be sure, is very peculiar in its hæmolytic behavior), for thus far no fitting complements have been found for this serum. In all other cases of hæmolysis through normal sera, which have been investigated for this purpose, according to our experiences positive proof of the presence of the amboceptors has been furnished.

The normal bactericidal sera also owe their bactericidal power

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<sup>1</sup> P. Müller, l. c.

to the co-action of two substances. Pfeiffer<sup>1</sup> furnished the first observations which led to this view when, in 1895, he succeeded in restoring the bactericidal power of inactivated goat serum in the peritoneal cavity of a guinea-pig. Moxter<sup>2</sup> subsequently demonstrated the presence of normal bacteriolytic amboceptors by means of reactivating experiments *in vitro*. And according to the numerous investigations of M. Neisser and Wechsberg in this Institute, all of the bacteriolysins of normal sera which they investigated, are of complex constitution. *This is natural because in the cell-destroying properties of normal serum, as in the development and increase of these properties through immunization the mechanism is exactly the same in principle, although, owing to the multiplicity of the reaction products, the action of the latter appears more complex.*

In my investigations of the *cytotoxic* properties of normal serum I have included the widely distributed *spermotoxic function*. According to the unanimous opinion of all authorities the specific spermotoxin produced by immunization consists of two substances. Thus far, however, this has not been demonstrated for normal spermotoxin, and Metchnikoff<sup>3</sup> has regarded the impossibility of reactivating the heated normal spermotoxic serum as an important diagnostic means to differentiate the latter from the specific immune serum. In opposition to this, by means of suitable mixtures, I was able, here also, to convince myself of the *complex nature of the normal spermotoxin*. After the spermotoxic property of rabbit serum for guinea-pig spermatozoa had been destroyed by heating to 56° C., I was able to restore this by the addition of guinea-pig or horse serum provided I mixed the inactive rabbit serum and guinea-pig serum in the proportion of 3:1 or 3:2. In that case the guinea-pig spermatozoa were killed after 12-15 minutes, whereas, in the control test with inactive rabbit serum or active guinea-pig serum alone, the spermatozoa showed lively movements even after 1½-1½ hours. The proportion of amboceptor and complement employed by me is in direct contrast to that recommended for immune sera by Metchnikoff and his co-workers. The reason for this will be under-

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<sup>1</sup> R. Pfeiffer, Weitere Mittheilungen über die Spezifischen Antikörper der Cholera, Zeitschr. f. Hygiene, XX, 1895.

<sup>2</sup> Moxter, Über die Wirkungsweise der bacterienauflösenden Substanzen der thierischen Säfte, Centralbl. f. Bacteriol., XXVI, 1899.

<sup>3</sup> Metchnikoff, Etudes sur la Spermotoxine, Annales de l'Institut Pasteur, 1900.

stood when the high degree of amboceptor concentration in immune sera is considered. In my case larger amounts of guinea-pig serum must be avoided, because in large doses the guinea-pig serum by itself finally exerts a toxic action on guinea-pig spermatozoa. This agrees with a statement of London (l. c.) that most all normal sera contain autospermotoxins.

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SUBSEQUENT NOTE.—In the meantime the French translation of a study by London, which had already been published in Russian, has appeared (*Contribution à l'étude des spermolysines*, Archives des Sciences Biologiques, T. IX, 1902), which shows that this investigator had also already recognized the complex constitution of the normal spermotoxin.

Our views concerning the complex nature of hæmoyslins have recently been confirmed by Flexner and Noguchi through the successful separation of amboceptor and complement in the cold (Snake venom in relation to hæmolysis, bacteriolysis, and toxicity, *Journal of Experimental Medicine*, vol. VI, 1902).

## XVIII. CONCERNING THE PLURALITY OF COMPLEMENTS OF THE SERUM.<sup>1</sup>

By Professor Dr. P. EHRLICH and Dr. H. SACHS.

THE continued study of the hæmolysins of blood serum has not only considerably extended our knowledge of the origin and mechanism of the immunity reaction directed against cells, but has revealed to us an unsuspected complexity of cellular metabolism to which the numerous protective bodies circulating in the blood owe their existence. It is probably everywhere conceded at the present day that the specific cytotoxins produced through immunization consist of two substances, amboceptor and complement; and we must regard it as proven that the cytotoxic substances in normal serum are also of complex constitution.<sup>2</sup> *A simple alexin action, in Buchner's sense, does not exist.* But even within the limits of this complicated field, Ehrlich and Morgenroth through their experimental work, have come to a further pluralistic conception, so that the closer analysis of the factors making up the cytotoxic function of a serum is enormously complicated. Thus it has been found in immunization with cells, that not merely a single kind of amboceptor is developed in the blood serum, *but that a large number of different types of amboceptors appear*, which vary both in their cytophile and complementophile groups. Furthermore, a number of facts and theoretical considerations (discussed in detail in the Sixth Hæmolysin communication) could be satisfactorily explained only by the assumption of a plurality of complements, and were absolutely irreconcilable with the unitarian assumption of only one complement in each serum.

After all this one might well regard the pluralistic conception as well founded, and abandon all further theoretic argument along this line. But Bordet,<sup>3</sup> the strongest supporter of the unitarian

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<sup>1</sup> Reprint from the Berl. klin. Wochenschr. 1902, Nos. 14 and 15.

<sup>2</sup> See the previous study.

<sup>3</sup> Bordet, Sur le mode d'action des sérums cytolitiques, etc. Annales de l'Institut. Pasteur, May, 1901.

character, in a recent work especially designed to refute the pluralistic view of the complements, has published a series of experiments, which in his opinion necessarily point to a simple alexin. Bordet's argument is based on the discovery of the interesting fact that blood corpuscles or bacteria treated with an inactive immune serum specific for themselves were able to deprive a normal active serum of all its complement activity.

Bordet sensitized blood corpuscles with appropriate ambocaptors, and then exposed them to the action of a freshly drawn normal serum. If now he waited for the occurrence of haemolysis and then added sensitized cells, bacteria, or blood corpuscles of different species, they remained totally unchanged, although the serum that had been used as complement was capable in its original condition of destroying these also. When fresh serum was first brought into contact with sensitized *bacteria*, similar results were obtained. The blood corpuscles subsequently added did not then undergo haemolysis.

*If such an action on one of the sensitive substrata has once taken place, the active sera, as a rule, are deprived of all their complement functions, from which Bordet concludes that the destruction of the most varied elements by one and the same serum must be due to a single complement.*

It must be acknowledged that these experiments, which we have been able to verify in numerous cases, at first sight seem to support Bordet's view. If one assumes that a certain serum A, which is capable of complementing two different bodies B and C, one bactericidal and the other haemolytic, contains only a single complement, Bordet's results would then most readily be explained by assuming that the two immune bodies are identical in their complementophile groups. In that case, of course, owing to the previous exercise of the one function, the available complement will have been used up, so that nothing is left for the exercise of its second function. But a closer examination shows us that *this view is an artificial one, and does not correspond to the facts observed.* For if it be assumed that this particular serum A contains two different complements, both of which can be absorbed by the ambocaptors B and C, Bordet's experiment will find an entirely different explanation. Now previous investigations<sup>1</sup> have shown that the artificially developed immune sera are not of simple constitution, but contain a number of different ambocaptors possessing different com-

<sup>1</sup> Ehrlich and Morgenroth, p. 56.

plementophile groups. To one, therefore, conversant with this conception, Bordet's conclusion cannot appear otherwise than forced. The unity of the complement would only then be demonstrated by Bordet's experiment if in the immune serum employed for absorption but a *single* complementophile group came into action, and not a *plurality* of groups.

Despite these objections raised against Bordet's evidence, and in spite of Ehrlich and Morgenroth's previous positive demonstration of the plurality of the complements, it seemed advisable, owing to the importance of the question, to enter once more upon a thorough investigation of the subject. We at first confined ourselves to the complements which effect the hæmolytic actions, and have been able to bring forward a large number of new and more conclusive proofs for the diversity of these complements in the same serum. These investigations have in part already been mentioned by Ehrlich at the Congress of Naturalists in Hamburg.

The method of the experiments was guided by the following considerations. If only a *single* complement is present in a certain serum, it follows that all the complement actions of this serum would be *weakened equally* by any given influence, chemical, physical, or thermic. If, on the contrary, our view of the *plurality of complements* is correct, it should be possible through appropriate experimental conditions to influence the serum in such a way that *only a part* of the complements will be destroyed, while others remain intact. Not only the *absolute* inhibition of the action of a few complements, but also marked *quantitative* differences in the impairment of the individual completions can only be satisfactorily explained by the assumption of different substances as carriers of these properties. A single complement would have all its functions impaired equally.

We have especially subjected the complementing property of goat serum to a thorough analysis, using for this purpose five different combinations which can be activated by goat serum. For simplicity's sake, we shall designate them by the following numbers:

Case I. Guinea-pig blood—inactive normal goat serum.

Case II. Rabbit blood—inactive normal goat serum.

Case III. Rabbit blood—inactive serum of goats immunized with rabbit blood.

Case IV. Ox blood—inactive serum of goats immunized with ox blood.



Case V. Dog blood inactive serum of goats immunized with dog blood.

The various means by which we have succeeded in a separation of the single complements are as follows:

1. Digestion with papain.
2. Partial destruction with an alkali.
3. Partial destruction by heating to 50° C.
4. Combination with blood-cells.

We discovered that invariably under the influence of papain digestion four complementing actions disappeared, or were more or less strongly diminished. Only a single complement remained intact, namely, that fitting the antibody developed in goat serum through immunization with rabbit blood.

In these experiments 20 cc. goat serum mixed with 3 cc. of a 10% papain solution were placed in an incubator in order to digest the complements. We found that the proper time to interrupt the digestive process was usually thirty to forty-five minutes later, when an examination<sup>1</sup> demonstrated complete preservation of the complements for Case III with complete disappearance or considerable diminution of the others. Of the large number of our experiments made in this connection three examples may be cited. See Table I.

TABLE I.  
DIGESTION OF GOAT SERUM BY MEANS OF PAPAIN.

Solvent Power of the Goat Serum.						
Example I.		Example II.		Example III.		
(a) Digested	(b) Normal	(a) Digested	(b) Normal	(a) Digested	(b) Normal	
Case I	0.5 moderate	0.25 complete	0.5 trace	0.15 complete	0.5 moderate	0.25 complete
Case II	1.0 trace	0.5 complete	1.0 0	0.25 complete	1.0 ft. trace	0.5 complete
Case III	0.2 complete	0.15 complete	0.15 complete	0.15 complete	0.15 complete	0.15 complete
Case IV	0.3 little	0.06 complete	0.3 little	0.07 complete	0.5 strong	0.08 complete
Case V	0.5 trace	0.06 complete	—	—	0.3 almost	0.05 complete

<sup>1</sup> In all our experiments the amount of blood used as a reagent was 1 cc. of a 5% suspension.



When the papain was allowed to act longer, the resistant complement III was also affected, so that usually after one and one-half to two hours digestion, the goat serum was entirely deprived of all its complements.

Treatment with alkali in place of papain digestion gave analogous results. We made use of soda and proceeded as follows: 10 cc. goat serum to which 1 cc. 7% soda solution had been added, were kept in the incubator for one and one-quarter hours, and then neutralized with hydrochloric acid. The solvent power was compared with a goat serum which by the simultaneous addition of soda and hydrochloric acid, had been brought to the same concentration of salt without having been subjected to the damaging influence of the soda.<sup>1</sup> (See Table II.)

TABLE II.  
DESTRUCTION OF THE GOAT SERUM BY MEANS OF SODA.

		Solvent Power of the Goat Serum			
		(a) After Soda Treatment		(b) Normally	
Case	I	0.5	0	0.1	complete
"	II	1.0	0	0.6	"
"	III	0.12	complete	0.03	"
"	IV	0.5	0	0.04	"
"	V	0.3	0	0.04	"

Hence, owing to the action of the soda the complements for Cases I, II, IV, and V have completely disappeared, whereas Complement III is still present, although its action is but one-fourth of what it was.

We have furthermore effected a separation of the complements by heating the goat serum to 49°-50° C. for half an hour. At this temperature the solvent action of normal goat serum for rabbit and guinea-pig blood has been completely destroyed or almost so. On the other hand, the complement action for the artificially produced immune bodies is more or less preserved, as can be seen from Table III.

The experiment shows that in this case complement IV is the most resistant, in contrast to its behavior with papain or soda. In the two latter cases, complement III had shown itself the most resistant. If we examine the table more closely we shall further see a

<sup>1</sup> The resulting salt concentration, by the way, is so slight that the solvent power was in no way decreased thereby.

TABLE III.  
HALF AN HOUR'S HEATING OF THE GOAT SERUM TO 50° C.

	Solvent Power of the Goat Serum.		What is Left of the Original Solvent Power.
	(a) Heated.	(b) Normally.	
Case I	1.0 trace	0.1 complete	} almost nothing
" II	1.0 "	0.25 "	
" III	0.08 complete	0.01 "	
" IV	0.035 "	0.035 "	
" V	0.75 "	0.02 "	

difference in the diminution suffered by complement V and that suffered by complement III. This is so marked that merely a combination of the above three experiments already furnishes *positive proof that the complement actions in III, IV, and V proceed independently of one another, and are effected by three different complements.*

But against this method of proof the objection might be made that in the end we may still be dealing with simple [*einheitlich*] complements and that the results of the experiments mentioned do not necessarily indicate a plurality of complements. It could be assumed that the view we have expressed concerning the plurality of the complements was true only in a certain restricted sense. Thus it would be possible that the complements possessed but one haptophore group, but a *plurality of zymotoxic groups* of which *one* effected the damaging action in any individual case. It could then easily be imagined that the various zymotoxic groups differ from one another in their behavior toward chemic or thermic influences, so that perhaps one was injured by papain, and another by an alkali. In order to decide this possibility either one way or another it seemed advisable to undertake absorption experiments. In case of a simple complement with different zymotoxic groups, the complement would be absorbed as a unit, whereas in the other case, differences such as we have already observed on heating, etc., would be expected to occur.

Because of the great significance of absorption, we regard these experiments as particularly valuable. Our first experiments were made to see if the complements, like so many bodies known to chemistry would adhere to granular substances of various kinds by virtue of surface attraction. Bone charcoal, skin powder, lycopodium,

and diatom earth, which we employed for this purpose, all proved more or less unsuitable for the absorption of complement. A stronger absorbent power on the other hand was exhibited by organized materials, thus confirming the statements of von Dungern.<sup>1</sup> Suspensions of staphylococci, when used in sufficient quantity, were able to abstract the complements quite energetically.<sup>2</sup> In like manner yeast powder is an excellent means to deprive a serum of its complement properties. A separation of the complements, however, was not achieved by these experiments.

We are inclined to believe that in these cases the fixation of the complements is due to physical absorption and not to definite chemical union. This view is the outcome of the positive results obtained in the absorptions when we employed blood-cells which had been mixed with suitable amboceptors, and which, according to our views, were able to bind complements chemically. If blood-cells which have been saturated (sensitized) with a normal immune body or with one artificially produced are shaken with a certain amount<sup>3</sup> of complementing serum, it is very easy to determine that in conformity with the results of Bordet's experiments, the complement properties possessed by the normal serum have in most cases completely disappeared with the onset of hæmolysis. It was just this phenomenon that led Bordet to his unitarian conception. Yet even in this absorption it is possible by means of suitable methods to convince one's self of the diversity of the complements, for by making the time as short as possible *only those complements are absorbed which possess the strongest affinity for corresponding complementophile groups*. Naturally experiments of this kind are difficult and require considerable variation. But it is usually possible to finally devise a suitable method of procedure. An interesting case studied by us in this respect is the combination rabbit blood and goat serum (Case II). With sufficiently rapid digestion (2 to 3 minutes at the most, possibly with the aid of gentle heat) the decanted portion showed a considerable loss of complements for Case IV or V, or for both, without suffering any injury in the rest of its complement

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<sup>1</sup> See p. 36.

<sup>2</sup> The same results were obtained by Wilde (Berl. klin. Wochenschr. 1901, No. 34) in absorption tests with anthrax, cholera, and typhoid bacteria; but to conclude from this that the alexin is a simple unit, as Wilde does, is not permissible in view of our above statements.

<sup>3</sup> This amount must be determined separately for each case.

functions. We were able to observe this behavior repeatedly and reproduce the following as an illustration.

10 cc. goat serum are shaken with 8 cc. rabbit blood for a very short time and then rapidly centrifuged. The following table shows the solvent power of the decanted fluid and of normal goat serum. The figures, I-V, correspond to the blood-cell ambceptor combination employed in the previous tables.

TABLE IV.  
BRIEF ABSORPTION OF GOAT SERUM WITH RABBIT BLOOD.

	Solvent Power of the Goat Serum.	
	(a) After the Absorption.	(b) Normally.
Case I	0.25 complete	0.25 complete
" II	0.5 "	0.5 "
" III	0.04 "	0.04 "
" IV	0.35 complete	0.08 complete
" V	0.2 "	0.03 "

Complements I, II, and III have been completely preserved, IV and V have been reduced to one-fourth and one-seventh respectively, thus furnishing another proof for their diversity. It is of special interest that in this brief action the particular activating principle (complement II) which we shall term the "dominant complement" has not at all combined with the cell, whereas other complements, which are of no consequence so far as the solvent process is concerned, have already been subjected to a distinct absorption.

With the absorptions are also to be classed the experiments concerning Case I, which we have made with guinea-pig blood stromata obtained after the method of H. Sachs<sup>1</sup> by heating the blood to 55° C. In these stromata the receptors which bind the ambceptors present in normal goat serum have been preserved capable of reacting.

These experiments demonstrated the absorption of the complements for the two normal hemolysins (Cases I and II) while the rest of the complements were in the main preserved.<sup>2</sup> An experiment of this kind is shown in Table V.

<sup>1</sup> See page 167.

<sup>2</sup> In this also it is necessary first to determine the favorable conditions governing the experiment. Thus, in order to completely bind the guinea-pig

20 cc. goat blood are treated with the stromata from 53 cc. guinea-pig blood. After absorption has occurred the mixture is centrifuged and the complement action of the fluid compared with that of normal goat serum. (See Table V.)

TABLE V.

ABSORPTION OF THE GOAT SERUM BY GUINEA-PIG BLOOD STROMATA.

	Solvent Power	
	(a) Of the Decanted Fluid.	(b) Of the Normal Goat Serum.
Case I	1.0 faint trace	0.15 complete
" II	1.0 " "	0.25 " "
" III	0.1 complete	0.1 complete
" IV	0.15 complete	0.04 complete
" V	0.15 complete	0.15 complete

Hence after the absorption, the complements of the normal hæmolysins had almost completely disappeared, while complements III and V were entirely preserved. Complement IV occupies a place between these, for in this case also a partial absorption could not be avoided. Its behavior very prettily confirms the demonstrational ready made by us of this complement's peculiar isolated position.

Entirely analogous results are obtained when, instead of using guinea-pig blood stromata, a series of experiments is made with red blood-cells, using the red fluid obtained when the red blood-cells have dissolved directly as complement for another combination. In such experiments we could show that the blood solution thus obtained had lost complements I and II and contained only the complements for cases III, IV and V. This method of procedure

blood hæmolysin (amboceptor+complement) of normal goat-blood serum, it is necessary to absorb with a large excess of guinea-pig blood stromata. It then readily happens that some complements other than those belonging to the two normal hæmolysins suffer injury to a greater or less extent. This was observed especially in several cases in which, in order to render easier the complete binding of the complements for the *normal* hæmolysins, the guinea-pig blood stromata had been sensitized with a large amount of inactivated normal goat serum. In that case, evidently, several partial amboceptors present in the goat serum in relatively small amounts and possessing affinities also for the other complements come into play.

therefore confirms the separation effected by means of the stromata, whereby the complements of the normal hemolysins I and II are separated from the rest.

Bordet himself, by the way, has described such a case concerning the combination rabbit blood-guinea-pig serum. This experiment, of course, was not to be reconciled with his unitarian view, and he therefore sought to explain this inconvenient result in accordance with his view by assuming a special law of distribution for the normal hemolysins, together possibly with an inhibiting action exerted by the products of the destruction of the red blood-cells first used, on further solution of the same.<sup>1</sup> Against this we should like to emphasize that in our case the result has been confirmed by the experiment with blood stromata. By means of this, since the stromata plus the anchored complement is removed by centrifuging, Bordet's assumptions can be entirely excluded.

Our absorption experiments therefore show that *of the two possibilities, namely, of a complement with several different zymozic groups, or of a plurality of different complements, the latter assumption must be accepted.*

Regarding the number of complements to be assumed for normal goat serum, as based on our experiments, this can best be seen from the following table:

TABLE VI.

		Complementing Power of Goat Serum after					
		(a) Digestion with Papain.	(b) The Action of Soda.	(c) Heating to 500.	(d) Absorption with Held's Blood.	(e) Absorption with Guinea-pig Blood.	(f) Absorption with Guinea-pig Blood Stromata.
Case	I	0	0	0	+	0	0
"	II	0	0	0	+	0	0
"	III	+	+	+	+	+	+
"	IV	0	0	+	+	+	+
"	V	0	0	+	+	+	+

<sup>1</sup>This objection, moreover, is entirely incomprehensible to us. According to our view, normal and artificially produced hemolysins manifest their action by means of the same mechanism; for when the normal amoceptors are replaced by the host of amoceptors present in an immune serum, new complementophile groups come into action, and with these, of course, new partial complements.

This shows us that the two complements I and II (normal haemolysins) cannot by these experiments be differentiated from each other, *that the other three complements, however, can absolutely be distinguished by their behavior, not only from one another but also from the first group.* Hence in the five different combinations the existence of at least four different complements is positively demonstrated. And that the two normal haemolytic functions of goat serum are also effected by two different complements follows from a previous experiment of Ehrlich and Morgenroth.<sup>1</sup> These authors showed by filtering a normal goat serum through Pukall filters, that the filtrate contained exactly the same amount of complement for guinea-pig blood, whereas the complement for rabbit blood was almost entirely absent. E. Neisser and Döring<sup>2</sup> have confirmed this result in the case of human serum.

The necessary consequence, therefore, of our experiences with goat serum is the demonstration of the fact *that in the five combinations examined, five different complements of the goat serum come into play.*<sup>3</sup>

We have also examined the complementing properties of the sera of other animal species, and have arrived at results which absolutely contradict the unitarian view of the complements. These experiments concern first the serum of rabbits. We shall proceed from the fact determined by Schütze and Scheller<sup>4</sup> under Wassermann's direction, that, following intravenous injections of goat blood, the rabbit serum completely loses its property to dissolve goat blood.

The question now was whether the rabbit serum had been deprived merely of this one complementing function, or whether it had also suffered loss in the rest of its complement properties.

We therefore tested the power of rabbit serum, before and after the injection of goat blood, to activate the immune body obtained by immunizing rabbits with ox blood. As the essential result of our numerous investigations we established the fact that the com-

<sup>1</sup> See page 56.

<sup>2</sup> E. Neisser and Döring, Berl. Min. Wochenschr. 1901, No. 22.

<sup>3</sup> Through the courtesy of Dr. Wendelstadt in Bonn, we learn that that investigator, by means of an interesting method, has also succeeded in demonstrating a number of complements in goat serum. He immunized a goat with several species of blood and was then able by means of chemical and thermic influences to separate the complements fitting the immune bodies produced. See Centralblatt f. Bacteriologie, in which this study is about to appear.

<sup>4</sup> Schütze and Scheller, Experimentelle Beiträge zur Kenntnis der im normalen serum vorkommenden globulischen Substanzen, Zeitschrift f. Hygiene, Vol. 36, 1901.



plement for goat blood disappeared after the injection while that for the immune body sensitizing ox blood remained intact. The following test may serve as an example:

A rabbit of 1900 g. is injected intravenously with 22 cc. goat blood. The change in the solvent power of the goat serum which results from the injection may be seen from the following table:

TABLE VII.

Blood Species.	Solvent Power of the Rabbit Serum.	
	(a) Before the Injection.	(b) After the Injection.
Goat blood—inactive normal rabbit serum	0.35 complete	1.0 no solution
Ox blood—inactive serum of a rabbit immunized with ox blood. ....	0.05 “	0.25 complete

Similar results are obtained in the absorption of rabbit serum by means of goat blood *in vitro*, so that this experiment already justifies us in assuming two different complements in rabbit serum.

In one of these experiments with goat-blood injections the hæmolysis of pig blood by means of rabbit serum was also tested, and it was found that the complement of the normal hæmolysin for pig blood, like that for sensitized ox blood, had remained unchanged. Neither was it possible by means of intravenous injection of pig blood to separate these two complements of rabbit serum, for in this case, contrary to their previous behavior, both were absorbed, while the complement for goat blood remained in the serum. For the present we must therefore content ourselves with the knowledge that we have brought forward positive proof of *the existence of two different complements in rabbit serum; a proof which is strongly corroborated by the divergent behavior of the two complements in the absorption with goat blood and pig blood respectively.*

The difference between the two complements also manifests itself in their different vulnerability to papain. While the complementing power of rabbit serum toward the artificially produced immune body for ox blood suffers considerable diminution under the influence of papain digestion, the complement of normal hæmolysin for goat blood is hardly affected, so that this experiment also substantiates our demonstration of at least two complements in rabbit serum.

Some rather cursory tests were finally made with dog and guinea-



pig serum with the view of separating the complements by carefully heating the sera. In the dog serum a half hour's heating to 49.5° and in the guinea-pig serum to 49° was sufficient to enable us, by means of the differences of the weakening of the various complementing functions, to recognize here also the plurality of the complements. The results of these experiments are shown in Tables VIII and IX.

TABLE VIII.  
HALF AN HOUR'S HEATING OF DOG SERUM TO 49.5 C.

Blood-cell—Amboceptor Combination.	Solvent Power of the Dog Serum.		Solvent Power Still Preserved.
	(a) Heated.	(b) Normal.	
I. Rabbit blood—inactive dog serum. . . . .	0.5    0	0.25 complete	0
II. Guinea-pig blood—inactive dog serum. . . . .	0.5    0	0.1    "	0
III. Sheep blood—inactive dog serum. . . . .	0.5    0	0.08   "	0
IV. Human blood—inactive serum of goats immunized with human blood. . . . .	0.5 moderate	0.15   "	less than $\frac{1}{2}$
V. Ox blood—inactive serum of goats immunized with ox blood. . . . .	0.35 complete	0.06   "	$\frac{1}{2}$
VI. Ox blood—inactive serum of rabbits immunized with ox blood. . . . .	0.5 strong	0.045   "	less than $\frac{1}{3}$

TABLE IX.  
HALF AN HOUR'S HEATING OF THE GUINEA-PIG SERUM TO 49° C.

Blood-cell—Amboceptor Combination.	Solvent Power of the Guinea-pig Serum.		Solvent Power Still Preserved.
	(a) Heated to 49°	(b) Normal.	
I. Rabbit blood—inactive guinea-pig serum. . . . .	1.0    0	0.5 complete	0
II. Ox blood—inactive guinea-pig serum. . . . .	0.5    trace	0.5    "	almost 0
III. Ox blood—inactive serum of goats immunized with ox blood. . . . .	0.008 complete	0.008   "	1
IV. Ox blood—inactive serum of rabbits immunized with ox blood. . . . .	0.025   "	0.025   "	1
V. Sheep blood—inactive serum of goats immunized with sheep blood. . . . .	0.025   "	0.006   "	$\frac{1}{2}$
VI. Dog blood—inactive serum of goats immunized with dog blood. . . . .	0.5    "	0.25   "	$\frac{1}{3}$

If we review all our observations, they show that in the question of the complements the unitarian conception leads to a confused mass of inexplicable contradictions, and that it must therefore be abandoned. *All experiences, on the other hand, harmonize best with the assumption of a number of different complements in the same serum.* Sober consideration, in fact, makes this appear as the necessary consequence of such a multiplicity as has been demonstrated anew by these experiments. It is a satisfaction to know that in the Institut Pasteur a high authority (Metchnikoff)<sup>1</sup> has also given up the Buchner-Bordet conception of the simplicity [einheitlichkeit] of the alexines, and has come to the conclusion that there are at least two complements in the same serum. Metchnikoff found that the exudates rich in *macrophages* acted hæmolytically, but were unable to effect bacteriolysis. On the other hand the exudates rich in *microphages* exerted a marked bactericidal action, but were incapable of dissolving even sensitized red blood-cells. Metchnikoff concludes that these two kinds of cells produce two different complements, one, which he terms *microcytase*, effects the bacteriolytic actions, the other, *macrocytase*, is the carrier of the functions which destroy animal cells. He emphasizes that the demonstration of the duality of complements does not affect the correctness of Bordet's experiments, and he says in explanation of Bordet's results: "Il n'y a qu'à admettre que les éléments figurés, une fois qu'ils sont imprégnés de fixateurs spécifiques, deviennent capables d'absorber non seulement la cytase qui les digère, mais aussi une autre qui, sans les dissoudre, se fixe simplement sur eux."

So far as this is concerned we should like again to emphasize that we also have not questioned the correctness of Bordet's experiments, *but have merely objected to the unitarian conception deduced therefrom. The old controversy concerning the two views would thus be ended, and definitely decided in favor of our view.*

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<sup>1</sup> Metchnikoff, L'Immunité dans les maladies infectieuses, page 206, Paris, 1901.

## XIX. CONCERNING THE MECHANISM OF THE ACTION OF AMBOCEPTORS.<sup>1</sup>

By Prof. Dr. P. EHRLICH and Dr. H. SACHS.

### I. Blocking of the Amboceptor by Complementoids.

THE complements which activate the amboceptors of blood serum are, as is well known from the experiments of Ehrlich and Morgenroth, like the toxins characterized by two groups in the molecule, viz., the *haptophore group*, which combines with the complementophile group of the amboceptor, and the *zymotoxic group*, which represents the actual carrier of the complement's specific function. In complete harmony with this, Ehrlich and Morgenroth<sup>2</sup> could show through the production of anticomplements by heating inactivated sera, that the complements, like the toxins, under certain circumstances are changed into inactive modifications. These modifications are still able to excite the production of antibodies, and must therefore possess their haptophore group intact; in analogy with the toxoids, therefore, they are called *complementoids*. Although the presence of the complementoids could easily be shown by means of animal experiments, it was impossible to demonstrate their reacting power by means of hæmolytic test-tube experiments. The reason for this was that a decrease of the complement action, such as was to be expected in the inactivated sera (which really constitute a mixture of amboceptor and complementoid), did not occur, even when the complementoid was present in large amounts. Ehrlich and Morgenroth have therefore assumed that in the change from complement to complementoid, *the affinity of the complement's haptophore group suffers a diminution*. A similar assumption has been made by Myers<sup>3</sup> for the toxoids of cobra poison.

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<sup>1</sup> Reprint from the Berl. klin. Wochenschr. 1902, No. 21.

<sup>2</sup> See page 79.

<sup>3</sup> Myers, Cobra Poisons, etc., The Lancet, 1898.

It is, of course, not at all necessary that such a diminution of affinity occur with all complements; and, considering the great distribution and multiplicity of the substances included in the concept "complement," this is *a priori* but little probable. We have therefore hoped that in the course of our investigations we would discover a suitable combination in which, on the formation of complementoid, the diminution of affinity does not occur, or occurs only to a slight degree. As a matter of fact, such a case has recently presented itself to us.

As is well known, normal dog serum dissolves guinea-pig blood energetically. If this dog serum is inactivated, it is easy to restore the hemolytic property with active guinea-pig serum; the inactivation, however, must be effected at suitable temperatures, 50–51°C., for at higher temperatures, as Sachs<sup>1</sup> has demonstrated, the ambocceptor of dog serum shows itself thermolabile.

That is why Buchner in his experiments could not activate the ambocceptor of dogs, for at the inactivating temperature employed by him, 60°C., the completion with guinea-pig serum is no longer possible. Continuing the analysis of this interesting case we made a curious observation: If guinea-pig blood-cells were treated with appropriate amounts of inactive dog serum for one hour in an incubator and the mixture then centrifuged, it was found that, contrary to all expectations, the sediments could no longer be activated with guinea-pig serum, whereas when the three constituents were mixed simultaneously, prompt hemolysis occurred. (See Table I.)

Our first thought was that the ambocceptor, despite the relatively long contact with the blood-cells (one hour), had perhaps not been bound by these. Such behavior, to be sure, although conceivable and, as we shall see later, sometimes actually occurring, would be exceptional. In this case, however, we could readily convince ourselves that this suspicion was groundless. For when by means of guinea-pig serum we attempted to activate the guinea-pig blood-cells digested with dog serum as above described, *without first removing the fluid medium*, no hemolysis took place. And we could see by the behavior of the fluid obtained by centrifuging the blood mixture as described that the ambocceptor was not present in the fluid. When this was allowed to act on native guinea-pig blood to which active guinea-pig serum (complement) had been added, no solution could

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<sup>1</sup> See pages 181 et seq.

TABLE I.

Inactive Dog Serum.		Solvent Action on the Guinea-pig Blood.	
		(A)	(B)
cc.		Blood + Inactive Dog Serum kept at 37° for One Hour, then Centrifuged To the Sediment 0.5 cc Guinea-pig Serum.	Blood + Inactive Dog Serum + 0.5 cc Guinea-pig Serum Mixed Simultaneously
1.	1.0	0	complete
2.	0.5		"
3.	0.35		"
4.	0.25		"
5.	0.15		almost complete
6.	0		0

<sup>1</sup> The amount of blood used in our experiments is always 1 cc. of a 5% suspension in .65% salt solution.

be effected. *Hence the amboceptor must have been bound by the blood-cells.*

How then, through this previous binding, had the amboceptor lost its power of being activated? After excluding all other possible explanations we were forced to conclude *that the phenomenon observed is due to a blocking of the complementophile groups of the dog serum's amboceptor by the complementoids still present in the inactive serum.* The correctness of this view has to our minds been confirmed:

1. By the isolated binding of the amboceptor at 0° C.
2. By the subsequent blocking of the amboceptor bound at 0° C., by means of free complementoids.
3. By the behavior of dog serum inactivated by shaking with yeast.
4. By the combining experiment with inactive dog serum (inactivated by heat) when the salt content of the fluids was increased.

We shall take these up in order.

1. If we repeated the combining experiment above mentioned, modifying it, however, so that the amboceptor was anchored by the blood-cells, not at 37° C., but at 0° C., *we could show that the guinea-pig blood-cells, treated in this way at 0° C., were all activated by guinea-pig serum.* (See Table II.)

Now we know that at 0°, as a rule, only the amboceptor is bound by the blood-cells, and that the complement for the most part is uninfluenced. It is, therefore, perhaps quite natural in those cases in which the complementoids, like the complements, are bound by

TABLE II.  
GUINEA-PIG BLOOD.

Inactive Dog Serum.	cc.	Amount of Solution of the Sediments on the Addition of 0.4 cc. Guinea-pig Serum, after Previously having been Treated.	
		(A) At 0°.	(B) At 37°.
1.	1.0	complete	0
2.	0.5	"	
3.	0.35	"	
4.	0.25	"	
5.	0.15	almost complete	
6.	0	0	

the amboceptors, that this binding will not take place if the experiment is performed at 0° C. These considerations confirm our view that the impossibility of activating the blood-cells sensitized at 37° C. is due to a blocking of the complementophile amboceptor groups of the dog serum by the complementoids of the same serum.

2. It still remained to show that the substance which prevented the binding subsequent to the binding effected at 0° C., was really present in the fluid medium. This could easily be shown in the following manner. Two parallel series of tubes with guinea-pig blood were treated at 0° C. for one and one-half hours with inactive dog serum (i.e., containing amboceptor + complementoid). The tubes of series A were then centrifuged and the sediments, freed from fluid, suspended in physiological salt solution; the tubes of series B were left unchanged. All the tubes were now placed into the incubator for one hour, then centrifuged, and the sediments mixed with active guinea-pig serum and physiological salt solution. In the tubes of series A solution ensued, the blood-cells of series B remained undissolved, as can be seen from Table III.

*The substance which caused the blocking of the amboceptors was therefore contained in the fluid portion of the blood sensitized at 0°; for in series A, in which the fluid medium was decanted, the blood-cells although subsequently kept at 37° C., could still be activated. In series B, on the contrary, the complementoids still remaining free at 0° C., were bound when subsequently kept in the thermostat, and so prevented the "completion" with active serum. From all this it follows that we can be dealing only with complementoid action in the test-tube, and the correctness of this view is confirmed in another way.*

TABLE III.

Inactive Dog Serum.		Amount of Solution of Guinea-pig Blood on the Addition of 0.4 cc. Guinea-pig Serum.	
		Series A.	Series B.
1.	1.0	complete	} 0
2.	0.5	"	
3.	0.35	strong	
4.	0.25	moderate	
5.	0.15	"	
6.	0	0	

3. We know from the studies of v. Dungern<sup>1</sup> and Ehrlich and Sachs,<sup>2</sup> that yeast constitutes an excellent means of removing the complements of a serum. If we prepared an inactive dog serum by treatment with yeast instead of with heat, or if we allowed the complementoids of a serum inactivated by heat to be absorbed by yeast, it was found that a dog serum so treated was no longer capable of causing this "blocking" phenomenon. Hæmolysis occurred in like manner whether we added the activating guinea-pig serum at once, or first kept the blood-cell—dog-serum mixture in the thermostat for an hour. (See Table IV.)

TABLE IV.

Dog Serum,		Amount of Solution of Guinea-pig Blood on the Addition of 0.4 cc. Guinea-pig Serum after Remaining at 37° C. for One Hour.		
		Dog Serum Inactivated.		
		(A)	(B) By Heating.	
cc.	By Shaking with Yeast.*	(a) Shaken with Yeast.*	(b) Employed Directly.	
1. 1.0	complete	complete	}	0
2. 0.5	“	“		
3. 0.35	“	“		
4. 0.25	almost complete	almost complete		
5. 0.15	strong	strong		
6. 0	0	0		

\* 6 cc. serum are shaken with 0.2 grams yeast.

*The complementoids had simply been removed by the yeast and the isolated amboceptors reacted in normal fashion.*

<sup>1</sup> See page 36 et seq

<sup>2</sup> See page 195 et seq.



4. A further proof of the correctness of our view was furnished by the results of the combining experiment when the molecular concentration of the fluid medium was increased. As is well known the hemolytic action of the sera is retarded and even entirely prevented by an increase in the amount of salts present. The investigations of Markl<sup>1</sup> have shown that under these circumstances the anisceptor is bound by the red blood cells, whereas the complement is unable to take hold.<sup>2</sup> Through extensive investigations, not yet published, we have been able to verify this. Under these circumstances, provided the view developed by us is correct, it should naturally be possible to prevent the blocking with complementoids by means of suitable concentrations of salts. Two parallel series of tubes with guinea-pig blood to which inactive dog serum had been added were therefore kept at 37°C. for one hour, ammonium sulphate having first been added to one of the series in the strength of 1.5%. This addition, as special tests showed us, suffices to entirely prevent the hemolytic action even of large amounts (1 cc.) of active dog serum. The result of the experiment corresponded exactly to our expectations. The sediments of those guinea-pig blood cells which had been treated with ammonium sulphate could be complemented with guinea-pig serum, whereas in the other series no solution whatever occurred. (See Table V.)

*The analysis of this case furnishes the first proof by means of test-tube experiments that complementoids, the inactive modifications of the complements, actually exist in the inactive serum. To be sure, even heretofore their existence could not appear doubtful, for, in our opinion, through the possibility of producing antibodies, proof had been furnished of the preservation of the complement's haptophore group in the inactivated serum.<sup>3</sup>*

<sup>1</sup> Markl, Über Hemmung der Hämolyse durch Salze. *Zeitschr. f. Hygiene*, Vol. 39, 1902.

<sup>2</sup> These conditions by the way, in our judgment, have no connection with the osmotic conditions of the cell membrane, as Markl believes. It seems to us that the action of the salts is most readily explained by assuming that the increased concentration hinders the chemical union of anisceptor and complement. That the salts can exert such an antireactive action is seen by the fact pointed out by Kneer (*Münch. and Wochenschr.* 1898, Nos. 11 and 12) that tetanus antitoxin and toxin are absolutely prevented from combining by the addition of 10% NaCl.

<sup>3</sup> In view of this new confirmation I should not want to deprive the reader of an exposition of the complementoid theory from the standpoint of an opponent



TABLE V.

Inactive Dog Serum.	Amount of Solution of the Guinea-pig Blood Sediments Centrifuged, after the Mixtures had been kept for One Hour at 37°C. on the Addition of 0.5 cc. Guinea-pig Serum, the Mixtures having been Previously Treated with		
	cc.	(A) 0.15 cc. 20% $(\text{NH}_4)_2\text{SO}_4$	(B) 0.15 cc. 0.85% NaCl
1.	1.0	complete	
2.	0.5	moderate	
3.	0.35	little	0
4.	0.25	trace	

In contrast to the usual behavior we must assume that in the case described the *affinity of the complement has not suffered any considerable decrease through the formation of complementoid*. This is supported also by an experiment which we made in order to determine the lowest temperature at which the anchoring of the com-

(*Protocoll der k.k. Gesellschaft der Aerzte in Wien, Wiener klin. Wochenschrift, 1901, No. 51*):

"If an animal is injected with inactive serum of the same foreign species instead of active serum, it is found that its serum likewise becomes charged with anticomplement; proof that the alexin also—like everything else in this world—contains a haptophore group and an active group, the latter this time termed zymotoxic. As a result of the inactivation the zymotoxic group is destroyed; the haptophore group remains intact. Hence a continuance of the assimilation of complementoid and the production of the anticomplement. So far, so good. Now, however, we come to a questionable point. If the complement deprived of its zymotoxic group still possesses its haptophore group, it must still be able to satisfy and bind its amboceptor. How then does it happen that an inactivated antiserum again becomes lytic on the addition of suitable complement (active normal serum), a phenomenon which, according to Ehrlich (despite Dr. Wechsberg), is due to the formation of lysis from amboceptor and complement. If the haptophore group of the amboceptor has already been bound by the remains of the old complement, the 'complementoid,' it surely is unable to bind new complement. Hence by heating (inactivating) the serum the haptophore group of the complement cannot have remained unchanged; it must have completely lost its affinity for the amboceptor. Now, gentlemen, I should like to know what is left of the complement after this heating? The zymotoxic group is destroyed, the haptophore group so changed that it is not recognizable. Nothing remains of the complement except Ehrlich's fervent wish that a little of it might be left, because otherwise it would not harmonize with the theory! It is this wish that floats around in the inactive serum under the name of complementoid."

Thus far Gruber! I shall refrain from any personal remarks for which the

plementoid still takes place. In this way we sought to find an approximate criterion for relative affinity of the complementoid. From the power to be reactivated possessed by the guinea-pig blood cells previously treated at different temperatures with inactive dog serum it was seen that even at 3° C. a moderate binding of complementoids takes place, and that *complete blocking phenomena* can already be obtained at 8° C., as is seen in the following experiment:

TABLE VI

Inactive Dog Serum.	Amount of Inhibition of Guinea-pig Blood on the Addition of 0.5 cc. Guinea-pig Serum after Preliminary Treatment at			
	(A) 0° C.	(B) 3° C.	(C) 6° C.	(D) 8° C.
1. 1.0	complete	moderate		
2. 0.75	almost complete	"	faint trace	0
3. 0.5	strong	"		
4. 0.35	moderate	little		

Nevertheless we believe that even in this case a certain, though slight, decrease in the affinity has occurred in the complementoid formation. At least the fact speaks in favor of this, that with the simultaneous addition of inactive dog serum (i.e., ambceptor + complementoid) and active guinea-pig serum solution of the guinea-pig blood occurs. Under these circumstances, in which the ambceptor has both complement and complementoid to choose from, the former is preferred. When, then, we find that it is possible by previous treatment with complementoid to block the complementophilic group of the ambceptor for the complement subsequently added, we shall explain this most readily by assuming that *after the complementoid has been anchored, the union becomes firmer*. Analogous phenomena are common in immunity. Thus Donitz<sup>1</sup> has shown that the union

unusual tone of this attack surely offers sufficient provocation, merely expressing my astonishment at the fact that Gruber's exposition disregards the most important and explanatory point, namely, as Morgenthau and I have emphasized, that in the change into complementoids, the complements must usually suffer a decrease in their affinity, for only in this way can the absence of all disturbing interference on the part of these complementoids in test-tube experiments be explained. If, however, Gruber assumes a complete destruction of the complements by inactivation how does he explain the fact easily verified by every one, namely, the production of anticomplements by injection into the organism of serum which has been heated? Surely a mere wash floating around in the serum cannot suffice to produce anticomplements.—Ehrlich.

<sup>1</sup> Donitz, *Über die Grenzen der Wirksamkeit des Diphtherieheilserums*. Arch. internat. de Pharmacodyn., Vol. V, 1899.

of diphtheria poison in the animal body, at first a loose one, soon becomes more and more firm so that it cannot be broken up even by very large amounts of antitoxin. Madsen's<sup>1</sup> experiments, to liberate, by means of antitoxin, tetanolysin which had been anchored by the blood-cells, also confirm this.

Blocking by means of complementoids is also of value for the technique of demonstrating the presence of amboceptor. Suppose, for example, that in doubtful cases one seeks to show the existence of the amboceptors in the usual manner, by sensitizing the red blood-cells and subsequently complementing with a different kind of serum. In this case, owing to the blocking action of the complementoids, an absence of the amboceptors could be simulated. In this connection it is of considerable interest to know that so capable an investigator as Buchner<sup>2</sup> employed the above method for analyzing the hæmolysin in just the case here described. His attempts to demonstrate the amboceptor by this method (inapplicable in this particular instance), as well as by means of the amboceptor's thermolability<sup>3</sup> (already discussed), were unsuccessful.

## II. Amboceptor or Sensitizer?

In another case we have met with a different complication equally fatal to the successful demonstration of the amboceptor by routine procedures. This is of especial interest for the theory of hæmolysin action, and concerns the hæmolytic property of ox serum for guinea-pig blood. If the ox serum is inactivated, this property can readily be restored by the addition of active horse serum. If, however, one tries by means of active horse serum to complement blood-cell sediments (obtained by centrifuging guinea-pig blood after

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<sup>1</sup> Madsen, Über Heilversuche im Reagensglas. *Zeitschr. f. Hygiene*, Vol. 32, 1899.

<sup>2</sup> H. Buchner, Sind die Alexine einfache oder complexe Körper? *Berl. klin. Wochenschr.* 1891, No. 33.

<sup>3</sup> According to the newer researches already mentioned it would be conceivable that the thermolability of the amboceptors is simulated by this,—that the complementoids, in themselves possessing a relatively high affinity, become firmly anchored to the amboceptors. However, as special experiments have shown us, such is not the case, for dog serum which has been inactivated by shaking with yeast, and which therefore contains no complementoid, likewise loses its ability to be activated when it is heated to 60° C. It does not lose this power when heated only to 50°–51° C.

these have been treated at 37° C. for one hour with inactive ox serum), it will be found, just as in the previous case, that hæmolysis does not occur.

The reason for the non-activatibility in this case differs essentially from that in the case previously described. The chief difference manifests itself in the behavior of the decanted fluid medium. If the centrifuging is omitted, and active horse serum is added to the sensitized blood-cells without previously removing the fluid medium, it will be found that solution occurs. If the centrifuging is not omitted, it will be seen that the decanted fluids behave in an analogous manner, for when mixed with active horse serum they will dissolve native guinea-pig blood. A complete experiment is reproduced in Table VII.

TABLE VII.

Inactive Ox Serum.	Amount of Solution of Guinea-pig Blood on the Addition of 0.5 cc. Horse Serum to:			
	(A) The Sediments on Centrifuging after the Mixture had been kept at 37° C. for One Hour.	(B) The Decanted Fluids from (A) Added to Native Guinea-pig Blood.	(C) The Uncentrifuged Mixture of Blood and Ox Serum.	
			(a) After Remaining at 37° C. for One Hour.	(b) Immediately.
cc.				
1. 0.5	faint trace 0	complete	complete	complete
2. 0.35		"	"	"
3. 0.25		"	"	"
4. 0.15		strong	{ almost	almost
5. 0.1		moderate	complete	complete
6. 0		0	strong 0	strong 0

In contrast, therefore, to the behavior in the first case described by us, the amboceptor has remained in the decanted fluid, and has therefore not been bound by the blood-cells, or only to a very slight degree. Our attempts by means of horse serum to activate the guinea-pig blood-cells which had previously been treated at 0° C. with inactive ox serum and then centrifuged, failed as a matter of course; and the result was the same when the ox serum had been freed of complementoid by shaking with yeast.

This peculiar behavior, namely, that the amboceptor by itself does not unite with the cell at all, and acts only after it has combined with the complement, is of special significance for the method

of analyzing haemolysins. For, entirely aside from the fact that under these circumstances the attempt to activate the centrifuged, and presumably "sensitized," blood-cells necessarily fails, it will be seen that the occurrence of this complication considerably limits the application of the second method employed to discover the complex nature of haemolysins, namely, separation in the cold, a method already markedly restricted. This method, it will be recalled, depends upon the fact that at 0° C. usually only the amboceptors are bound to the blood-cells, not the complements to the amboceptors. In the case just described, however, the union of amboceptor and cell depend on the combining of amboceptor and complement. How, then, can a separation of the two components be effected if, on the one hand, the *conditio sine qua non* for the union of amboceptor and cell, a condition which obtains here, cannot be fulfilled at low temperature; and if, on the other, it in itself precludes any separation whatever? No wonder, therefore, that Gruber<sup>1</sup> failed with the cold separation method in just this case (guinea-pig blood + active ox serum).

The two atypical cases here described are, however, peculiarly adapted to throw light on the mechanism of haemolysin action. In the first case the fact that blood-cells "sensitized" in the usual manner withstand the action of the complement is hard to explain in accordance with Bordet's view. But the behavior shown in the second case becomes entirely inexplicable if, like Bordet, we believe the action of haemolysins to consist in this, that the amboceptors (substance sensibilatrice) sensitize the blood-cells and so render them vulnerable to the action of the complements (Bordet's alexins) exerted directly upon them. For here we have demonstrated that a sensitization does not take place; the amboceptor by itself is not at all bound, and becomes effective only on the addition of complement. If, however, we were to assume that in our case the complement nevertheless attacks the cell directly so that then the amboceptor can be found, we should arrive at a theory as unlike Bordet's as that held by Ehrlich and Morgenroth. But such a theory, strange to say, would apply only to this and perhaps a few other cases, that is, only to a few exceptions. Although superfluous, a suitable experiment was also made in this case and, as might have

<sup>1</sup> Gruber, Zur Theorie der Antikörper. Münch. med. Wochenschr. 1901, No. 49. See also H. Sachs, l. c.

been expected, it was found that the complement as such was not bound by the cell.

The facts, however, are very readily explained if, following Ehrlich and Morgenroth, we regard the amboreceptor as a complex possessing two haptophore groups. Owing to a mutual combination this transmits the action of the complement to the cell. In the case just described, it follows at once that the cytophile group of the amboreceptor possesses a very slight affinity to the cell receptor. We have therefore only to assume that, in contrast to the usual behavior, the amboreceptor in this case, while itself unable to combine with the cell, by combining with the complement takes on increased affinity and so becomes capable of action.

The significance of the variations in affinity will be discussed connectively at a subsequent time. We shall content ourselves here by pointing out that an understanding of the phenomena of immunity is impossible without the assumption that certain haptophore groups become increased or decreased in their chemical energy, owing to changes in the total molecule. Chemically, such an assumption is a matter of course. We believe that the observations described above constitute additional proof that amboreceptor and complement combine with each other.

In the main this question has already been decided by the beautiful investigations of M. Neisser and Wechsberg<sup>1</sup> on the deflection of complement by an excess of amboreceptor. The objections raised against these experiments by Gruber<sup>2</sup> and by Metchnikoff<sup>3</sup> have been completely met by the recent investigations of Lipstein.<sup>4</sup>

The case last described by us is to a certain extent an *experimentum crucis* for the correctness of the views formulated by Ehrlich and Morgenroth for the mechanism of hemolysin action. We therefore believe that Bordet's sensitization theory has become untenable, and that now this question, just as that concerning the plurality of complements, is definitely closed.

**SUBSEQUENT ADDITION.**—According to recent investigations of Dr. Sachs, guinea-pig blood-cells, which, because of treatment with inactive dog serum, can no longer be dissolved by guinea-pig serum, owing to blocking by com-

<sup>1</sup> See page 120 et seq.

<sup>2</sup> Gruber, *Protocoll der k. k. Gesellschaft der Aerzte in Wien*, *Wiener klin. Wochenschr.* 1901, No. 50.

<sup>3</sup> Metchnikoff, *L'Immunité dans les maladies infect.*, page 313, Paris, 1901.

<sup>4</sup> See page 132 et seq.

plementoid, are still dissolved by the complements of dog serum. The source of the dog serum complement was the fluid decanted from guinea pig blood-cells which, by treatment with active dog serum at 0° C., had abstracted as much of the amboceptor from the latter as possible. These experiments therefore show:

1. That the complement of dog serum suffers a diminution of affinity in changing to complementoid.
2. That the complement present in guinea-pig serum possesses a weaker affinity than the complement of dog serum with analogous action.

## XX. DIFFERENTIATING COMPLEMENTS BY MEANS OF A PARTIAL ANTICOMPLEMENT.<sup>1</sup>

By H. T. MARSHALL, Fellow of the Rockefeller Institute, and Dr. J. MORGENROTH, Member of the Frankfurt Institute.

THE question whether the serum of one and the same species contains a plurality of complements or only a single one seems to us to have been positively decided in favor of the pluralistic conception. This decision has been brought about mainly by the observations of Ehrlich and Morgenroth,<sup>2</sup> of Wassermann,<sup>3</sup> Wechsberg,<sup>4</sup> Wendelstadt,<sup>5</sup> and by the recently published studies of Ehrlich and Sachs.<sup>6</sup> Nevertheless, we shall briefly describe an experiment which, in a single instance at least, constitutes a proof for the plurality of the complements. Our object in doing this is not that the number of arguments may be further increased, for they are already amply sufficient, but that we may call attention to a method of demonstration which has not heretofore been employed.

Because of purely technical difficulties the most rational and simplest method of differentiation, namely, by means of anticomplements, has not thus far been employed in this question. As is well known, it is very easy by immunizing with serum containing complement or complementoid to obtain potent anticomplements. Such anticomplement sera, however, usually contain anticomplements corresponding to the sum of all the complements originally injected,<sup>7</sup> and are, therefore, not adapted to the separation of complements.

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<sup>1</sup> Reprinted from the *Centralblatt f. Bact.* Original Vol. XXXI, No. 12, 1902.

<sup>2</sup> See pages 11, 56, 86.

<sup>3</sup> Wassermann, *Zeitschr. f. Hyg.*, Vol. XXXVII, 1901.

<sup>4</sup> Wechsberg, *Sitzung d. k. k. Ges. d. Aerzte in Wien, Wiener klin. Wochenschr.* 1901, No. 48.

<sup>5</sup> Wendelstadt, *Centralblatt f. Bact.* Part I, Vol. XXXI, No. 10.

<sup>6</sup> See page 195 et seq.

<sup>7</sup> See page 63 et seq.



At least this had been the case thus far, for a partial anticomplement, one acting only against a single complement, had not been observed.

Through the courtesy of Dr. Cnyrim we detained a normal anticomplement which possessed the desired properties, and we therefore gladly availed ourselves of this favorable opportunity to demonstrate, by means of the elective binding of anticomplement, the difference between two complements in one and the same serum, a difference that had not heretofore been demonstrated.<sup>1</sup>

This normal anticomplement was an ascitic fluid derived from a case of cirrhosis of the liver; it exerted a marked antihaemolytic action in one particular case. By means of an experiment we first determined that this action was due to the presence of an anticomplement and not of an anti-immune body. This showed us that the ascitic fluid exerted practically no influence on the anchoring of the immune body in question to the red blood-cells.

The serum whose complements we examined was guinea-pig serum, which activated two amboceptors obtained by immunization. These amboceptors were contained in the inactive serum of a rabbit, A, which had been immunized with ox blood; and in the inactive serum of a goat, B, which had been immunized with sheep blood. Corresponding to this, ox blood was used for case A, and sheep blood for case B. The inactive ascitic fluid does not dissolve these species of blood even after the addition of guinea-pig serum

To begin, we saturated ox blood-cells with the specific amboceptor by adding 0.01 cc. immune body A to each 1 cc. of a 5% suspension of the cells. This is about ten times the amount which on the addition of sufficient complement (0.1 cc. guinea-pig serum) effected complete solution. The mixture was placed in the incubator and frequently shaken. At the end of one hour it was centrifuged, the fluid poured off, and the blood-cells, loaded with amboceptor, suspended in salt solution. In exactly the same manner sheep blood-cells were treated with the inactive serum B, 0.2 cc. for each 1 cc. of the 5% suspension. On the addition of guinea-pig serum to these blood-cells, haemolysis ensued very quickly in the thermostat; *in both cases* it required 0.008 cc. guinea-pig serum to fully dissolve 1 cc. of the suspension, while 0.0065 cc. caused incomplete solution and 0.002

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<sup>1</sup> In the following, for the sake of simplicity, we shall speak only of two complements, whereas we wish here to remark that two groups of complements are probably to be understood, each group made up of a host of single complements which it is impossible thus far to analyze.

only a slight degree of solution. For the sake of clearness it was especially fortunate that the complementing amounts should happen to be identical in the two cases.

A parallel series of experiments was then undertaken with these two cases, as follows: Varying amounts of the guinea-pig serum were mixed each with 0.4 cc. of ascitic fluid inactivated at 56° C., and the mixtures kept at room temperature for half an hour, after which the binding was entirely completed.<sup>1</sup> Thereupon the blood-cells loaded with amboceptor were added. The result of these experiments is shown in the following table:

CASE A (OX BLOOD + AMBOCEPTOR).

Guinea-pig Serum Alone.	Guinea-pig Serum + 0.4 cc. Ascitic Fluid.
0.008 complete solution	0.1 almost complete
0.0065 vestige	0.08 "
0.005 strong	0.065 considerable
0.0055 considerable	0.05 fairly little
0.003 "	0.035 very little
0.0025 moderate	0.03 trace
	0.025 "
	0.02 0

CASE B (SHEEP BLOOD + AMBOCEPTOR).

Guinea-pig Serum Alone.	Guinea-pig Serum + 0.4 cc. Ascitic Fluid.
0.008 complete solution	0.008 complete
0.0065 almost complete	0.0065 almost complete
0.005 "	0.005 "
0.0035 strong	0.0035 strong

We see, therefore, that in case A the complement protects *completely* against  $2\frac{1}{2}$  times the complete solvent amount of complement, while the amount of serum required to effect complete solution increases more than twelve times. In case B, on the contrary, the complete solvent dose of guinea-pig serum remains unchanged, and the series proceeds just as though there had been no addition of anticomplement.

These experiments, which were repeated many times, therefore

<sup>1</sup> The union of complements and anticomplements, analogous to the behavior of certain toxins and antitoxins, is dependent on the time. Hence here also this had to be considered and sufficient time allowed for the mixture to act

show that the ascitic fluid contains an anticomplement<sup>1</sup> which fits into that complement which is activated by amboceptor A, whereas anticomplements for the complement of amboceptor B are absent. Hence we are justified in differentiating in guinea-pig serum at least two complements with different haptophore groups.

It may be hoped that continued investigations of normal body fluids will bring to light numerous other favorable cases which will make possible differences along the lines indicated. For although in normal serum the complication of haptins present, such as amboceptors, complements, complementoids, antiamboceptors, and anticomplements, is very great, the conditions here are certainly simpler than in the serum of immunized animals; for in the latter there are also present innumerable primary, and (owing to internal regulative processes) secondary reactive products.

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<sup>1</sup> Ehrlich and Morgenroth have discussed the nature of anticomplements at length in the Berl. klin. Wochenschr. 1901, No. 10. They conclude that the origin of these bodies is this, that foreign complements combine with the complementophile group of certain cell receptors. According to this view the anticomplements are nothing else than thrust-off *amboceptors* whose complementophile groups possess a higher affinity than is usually the case. It is curious, therefore, that Gruber, nine months later (Sitzg. der k.k. Ges. der Aerzte in Wien, Wiener klin. Wochenschr. 1901), presents this view, which had been recognized as a natural consequence of the receptor theory, as an entirely new objection *against* just this theory.

## XXI. CONCERNING THE COMPLEMENTOPHILE GROUPS OF THE AMBOCEPTORS.<sup>1</sup>

By Prof. Dr. P. EHRLICH and H. T. MARSHALL, M.D., Fellow of the Rockefeller  
Institute of Medical Research.

THE studies of the past year, especially the recent conclusive work of Ehrlich and Sachs,<sup>2</sup> show that we may regard it as definitely proven that, in contrast to the unitarian conception of Bordet, there is a plurality of complements in the serum.

This knowledge largely supplements our views concerning the mechanism of lysin action, and is in complete harmony with the principles of the amboceptor theory. The latter, in contrast to the untenable sensitization theory of Bordet, has become still more firmly established through the recent experiments carried out in the Institute by M. Neisser and Wechsberg,<sup>3</sup> Lipstein,<sup>4</sup> and Ehrlich and Sachs.<sup>5</sup>

If we consider that, as is shown especially by Bordet's experiments,<sup>6</sup> an amboceptor, after having been anchored by cellular elements, can almost completely rob a serum of its complement, and if, further, we regard what we now know about the plurality of complements, we shall of necessity be led to a view concerning amboceptors according to which an amboceptor is *capable of binding a number of different complements simultaneously*. Attention was called to such a possibility by Ehrlich and Morgenroth<sup>7</sup> when they stated: "Finally, it is possible that an immune body, besides one particular cytophile

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<sup>1</sup> Reprint from the Berl. klin. Wochenschr. 1902, No. 25.

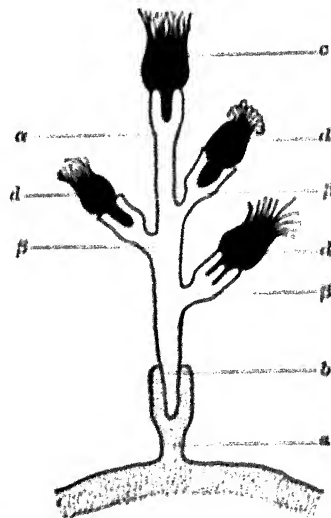
<sup>2</sup> See page 195.    <sup>3</sup> See page 120.    <sup>4</sup> See page 132.    <sup>5</sup> See page 209.

<sup>6</sup> Bordet, Annal. de l'Institut Pasteur, May 1901.

<sup>7</sup> See pages 88 et seq.

group, contains two, three, or more complementophile groups." According to this latter view, therefore, it is to be assumed that an amboceptor possesses *one* haptophore group specifically related to a certain receptor of cell or of a foodstuff, and that it also possesses a *number* of complementophile groups. The term amboceptor would thus indicate that two different substances, foodstuff and complement, are anchored by this body and brought into close relation with each other. This is illustrated in the following diagram.

FIG. 1.



(a) receptor of the cell; (b) haptophore group of the amboceptor; (c) dominant complement; (d) non-dominant complement.

Complementophile groups of the amboceptor: (α) for the dominant complement; (β) for the non-dominant.

The next question to be considered is *whether it is necessary, in order to get the specific lysis effect, for all these complements to come into action*. Recent experiments indicate that this is not the case, but that among the number of complements only a few are necessary in any single instance in order to obtain the effect. These complements are termed "*dominant complements*," the rest are "*non-dominant complements*."

A case described by Ehrlich and Sachs makes this clear, and we shall therefore briefly reproduce it here.<sup>1</sup>

Two ambocaptors are concerned, namely, the normal ambocaptor of goat serum for rabbit blood, and an ambocaptor obtained by immunizing goats, which is attached by ox blood cells. We shall for the sake of simplicity designate these ambocaptors as *A* and *B*.

Naturally both these ambocaptors are activated by goat serum, in which we shall have to assume at least two complements *x* and *b*. For immune body *A*, *x* is the dominant complement, for *B* it is *b*. If in one of the two combinations, for example, in that of rabbit blood cells loaded with immune body *A*, the serum is allowed to act long enough, both complements will be bound; that is, dominant and non-dominant. The result, however, is entirely different if the action be made as short as possible. In this case the fluid obtained on centrifuging the blood-cells still contains the dominant complement *x*, while it has for the most part lost the non-dominant complement *b*. We observe the surprising result that the immune body *A* with which the blood-cells are loaded combines with the non-dominant complement before it combines with its own dominant complement.

In this case, therefore, ambocaptor *A*'s complementophile groups which combine with the complement must possess a higher affinity for the non-dominant complement *b* than for the dominant complement *x*. Here then the binding of the non-dominant complement is independent of the binding of the dominant complement. Such a behavior, of course, is not a general rule; it was not long before a case was found in which the contrary was true, i.e., in which *the non-dominant complement does not combine until after the dominant complement has been bound*.

The demonstration of this relation succeeded only because in a certain human ascitic fluid an anticomplement was present which acted only against part of the complements of a serum. The peculiar behavior of this anticomplement has been described in a recent communication by Marshall and Morgenroth,<sup>2</sup> and is also readily seen in the following experiment. The complements here concerned are

<sup>1</sup> For the sake of clearness the case has here been somewhat simplified. The details of this experiment are found in Ehrlich and Sachs, page 195.

<sup>2</sup> See page 222.

present in normal guinea-pig serum. This serum reactivates two immune bodies, of which one, immune body *A*, was obtained by treating rabbits with ox blood, and the other, immune body *B*, by treating a goat with sheep blood. These immune bodies, naturally, acted respectively on ox blood-cells and sheep blood-cells. This *anti-complement* is strongly active in case *A*, while it is entirely without effect in case *B*. From this we may conclude that the complements concerned in these two cases, and which we may designate as  $\alpha$  and  $\beta$ , are unlike.

A further question was whether immune body *A* binds other complements in guinea-pig serum besides its own dominant complement. In order to determine this the following experiment was made: First, ox blood-cells and sheep blood-cells were saturated with their respective amboceptors *A* and *B*, and then to each cubic centimeter of the 5% blood suspension varying amounts of guinea-pig serum were added as complement. In the first case 0.0075 cc. guinea-pig serum sufficed to cause complete solution; in the second case 0.005 cc. was required.

Thereupon another test was made exactly like the preceding with ox blood and immune body *A*. After the mixture had remained in the thermostat at 37° C. for 1½ hours and hemolysis was practically completed, the same quantity of ox blood-cells laden with immune body (0.05 cc. ox blood freed from serum and made up to the original volume) was added anew and the mixtures kept in the thermostat for two hours longer. The hemolysis which had then taken place, observed by allowing the mixture to sediment in a refrigerator, indicated the amount of complement  $\alpha$  left after the first hemolysis and available for the case *A*.

At the same time a similar experiment was made in which, after the first hemolysis, sheep blood-cells saturated with amboceptor were used in the place of the ox blood-cells. In this case, after determining the amount of complement originally present, that of complement  $\beta$ , left after the first hemolysis, could also be found.

In this a considerable loss of complement is observed for both cases; for it now requires 0.075 cc. of the complementing guinea-pig serum to cause complete solution for case *A* and 0.025 cc. for case *B*, so that 1/10 and 1/5 respectively of the original complement are still preserved. This shows that the binding of complement  $\alpha$ , dominant for case *A*, is accompanied by a binding of complement  $\beta$ ,

dominant for case *B* but non-dominant for case *A*. It was necessary to determine whether or not in case *A* the absorption of the non-dominant complement  $\beta$  is dependent on the binding of the dominant complement  $\alpha$ . Owing to the peculiar nature of the complement it is possible to prevent the binding of complement for case *A*, whereas the binding of complement  $\beta$  for case *B* is not affected. On the addition of 0.4 cc. of the anticomplement serum the amount of complement necessary for complete solution increases from 0.0075 cc. to 0.2 cc., i.e. 26 times, whereas no such increase occurs for case *B*, 0.005 of the guinea-pig serum still causing complete solution.

If, therefore, the binding of the complement  $\beta$  by ox blood cells laden with amboceptor *A* is dependent on the binding of the dominant complement  $\alpha$ , it must be possible by the addition of anticomplement to prevent this binding. The following experiment is made as follows:

First, 0.4 cc. anticomplement serum is mixed with small amounts of guinea-pig serum. After this mixture has remained at room temperature for half an hour the ox blood-cells laden with amboceptor are added and the whole kept in the thermostat for 1½ hours, when the undissolved blood-cells are centrifuged off and the decanted fluid is mixed with sheep blood-cells loaded with their amboceptor. The result shows that in this case a decrease of complement for *B* has not occurred, for the tube containing 0.005 guinea-pig serum shows *complete* solution. The following table will make the results plain:

COMPLETE SOLVENT AMOUNTS OF GUINEA-PIG SERUM.

	I. Absolute Determination of the Complement.	II. After Binding the Complement by Means of Amboceptor + Blood-cells (Case <i>A</i> ).	III. After Binding the Complement by Means of 0.4 cc. Anticomplement	Amount of Complement (Amboceptor + Blood-cells) after the Addition of the Anticomplement
Case <i>A</i> .....	0.0075	0.075	0.2	—
Case <i>B</i> .....	0.005	0.025	0.005	0.

By means of this experiment, therefore, it has been proved that in this case binding of the non-dominant complement ensues on the corresponding complementophile group of immune body *A*.



chored the dominant complement  $\alpha$ . We shall probably not be wrong if we assume that in this case, owing to the occupation of the complementophile group for  $\alpha$ , there is an increase in affinity of the complementophile group for  $\beta$ . The subject of hemolysins contains many analogies for such a behavior. Thus it is quite common that not until the haptophore group of an amboceptor is bound to a cell does the complementophile group of the same possess sufficient affinity to anchor the complement.

Such an arrangement, whereby a single amboceptor is able to bind a number of different complements, is certainly not useless. Owing to their zymotoxic groups the complements can manifestly exercise quite different actions, so that the digestion of highly complex food molecules—in which, of course, we must see the physiological function of the amboceptor mechanism—is surely made easier. Such an arrangement seems still more adapted to the purpose when we consider that the cytophilic haptophore group of an amboceptor is fitted, not to the entire food molecule as such, but only to a partial group of the food molecule. The possibility is thus given for a particular amboceptor to anchor foodstuffs, which are almost entirely different but happen to agree in the possession of this one partial group. Granted that this is the case, the presence of only a single complement, acting only in one or the other possibility, would be dysteleological, whereas a plurality of complements would insure the greatest possible effect on the most varied foodstuff molecules. Recent investigations have brought to light a great many examples which show that in extracellular and intracellular digestive processes various ferments act together or in sequence. Thus, as Hofmeister<sup>1</sup> states, we already know of ten different ferments in the liver-cell: "A maltase, a glycose, a proteolytic ferment, a nuclein-splitting ferment, an aldehydase, a lactase, a ferment which converts the firmly bound nitrogen of amido acids into ammonia, a fibrin ferment, and, with some probability, a lipase and a rennin-like ferment." Even in so simple an organism as the yeast-cell, according to Delbrück,<sup>2</sup> at least five endoferments are demonstrable.

If one cares, one can regard an amboceptor whose various complementophile groups are occupied by different complements as a kind

<sup>1</sup> Hofmeister, Die chemische Organisation der Zelle. Vortrag Braunschweig, 1901.

<sup>2</sup> Delbrück, Jahrbuch des Vereins der Spiritusfabrikanten, Vol. II, 1902.

of *polyenzyme*. Analogous views have been expressed by Nencki<sup>1</sup> for the ferments of the digestive tract. Even though his conception, that pepsin is a single ferment with different active groups (pepsin group, rennin group, plasmin-forming group), does not entirely apply, we must say that his conception of such polyenzymes is fully justified. The properties of the ambocceptor above demonstrated will, we believe, speak in favor of the essential soundness of the view of this eminent chemist.

<sup>1</sup>Nencki and Sieler, *Zeitschr. f. physiol. Chem.* 1901.

## XXII. CONCERNING THE COMPLEMENTIBILITY OF THE AMBOCEPTORS.<sup>1</sup>

By Dr. J. MORGENSEN, Member of the Institute, and Dr. H. SACHS, Assistant  
at the Institute.

### I. A Presumptive Law Concerning the Complementibility of Normal Amboceptors and those Obtained by Immunization.

GRUBER<sup>2</sup> believes he has discovered an essential difference in the complementibility of the normal amboceptors of blood serum and those produced by immunization. He says: "The amboceptors<sup>3</sup> of the normal sera never seem to make the erythrocytes of another species sensitive to their own serum, . . . and I think I can say beforehand that the specific amboceptors regularly make the erythrocytes soluble in their own serum. This would constitute an essential difference between the two."

If Gruber believes Ehrlich has ever maintained that the amboceptors of normal and of immune sera are identical, this is a misunderstanding. On the contrary, the studies at this Institute<sup>4</sup> have emphasized that the immune sera, owing to the manifold variety of the reaction products developed in the immunization, contain a great host of different partial amboceptors whose cytophile and complementophile groups can vary greatly. Normal serum, on the contrary, possesses only few types of amboceptors identical with those of the immune serum. Hence if there is to be any question at all as to the identity of normal and artificially produced amboceptors, this can only be a partial identity. Special proof by Gruber of their non-identity in order to controvert the opposite view was therefore unnecessary. However, since what Gruber advances is incorrect and in

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<sup>1</sup> Reprint from the Berl. klin. Wochenschr. 1902, No. 27.

<sup>2</sup> Gruber, Munch. med. Wochenschr. 1901, No. 49.

<sup>3</sup> Gruber terms this "preparator."

<sup>4</sup> See especially pages 88 et seq.

contradiction to our experimental results, let us examine his evidence somewhat more closely.

In support of his first assertion, that "the antioceptor of the normal sera seems never to make the erythrocytes of another species sensitive to their own serum," he advances the following eight combinations (see Table I):

TABLE I

Number.	Species of Blood and Complement	Antioceptor
1	rabbit	ox
2	guinea-pig	"
3	rabbit	dog
4	guinea-pig	"
5	"	sheep
6	"	rabbit
7	"	chicken
8	rabbit	sheep

It seems entirely to have escaped Gruber that only a few lines previously he denies the existence of antioceptor in the first three of these combinations. Hence he should not have included these as evidences of the antioceptor's non-activability, for his own experiments had shown him that in these hemolyses no antioceptors could have been present<sup>1</sup>. From our own experiments we know that the next three combinations (4-6) usually lead to solution of the blood; there remain therefore only two cases (7 and 8) which we may consider as evidence of Gruber's contention<sup>2</sup>. Against these two cases can be placed a single case described by Gruber, one which he advances to support his second statement, "that the specific antioceptors make the erythrocytes soluble in their own serum." Gruber believes he can say in advance that this is regularly the case.

<sup>1</sup> Since then, however, antioceptors have also been demonstrated in these cases. (See H. Sachs, page 181.)

<sup>2</sup> One of these cases deals with the combination guinea-pig blood + chicken serum. From Ehrlich and Morgenroth's earlier communications (see pages 88 et seq.) Gruber could have seen that between animal species so far removed as chicken and guinea-pig the chances of complementability are not as great as they are between mammalian species. If Gruber therefore employs as evidence such distantly related species he must necessarily also have used widely separated species when complementing the immune sera. We have no doubt at all that by immunizing distantly related species (birds) with guinea-pig blood, antioceptors can be obtained which are not complemented by guinea-pig serum, or at least not regularly so.

Gruber has prophesied correctly. To one who has familiarized himself with the plurality of the amboceptors it will, to be sure, appear a matter of course that the erythrocytes loaded with specific amboceptors usually find suitable complements which cause their solution, as in most other sera, so also in their own serum. As a matter of fact, according to our own experience, the amboceptors of the immune sera seem as a rule to make the blood-cells sensitive to their own serum. But the far-reaching difference between the immune sera and normal sera which Gruber sees in this fact does not exist.

In the following table we have collected, either from personal knowledge or from the statements of other authors,<sup>1</sup> those cases in which the combinations blood-cells *a* + inactive normal serum (amboceptor) *b* + complement *a* lead to hemolysis, in contradiction to their behavior as stated by Gruber. (See Table II.)

TABLE II

Number.	Species of Blood and of Complement	Amboceptor.
1	guinea-pig	dog
2	"	calf
3	goat	rabbit
4	sheep	"
5	guinea-pig	sheep
6	"	horse
7	"	ox
8	rabbit	"
9	"	man
10	guinea-pig	rabbit

This table, which makes no pretense at completeness, shows that the solubility, in their own serum, of blood-cells loaded with normal amboceptor is quite common. This becomes still more evident when we consider that the combinations mentioned include only a limited number of the most common experimental animals, and that by using other species still more combinations would be found.

Gruber's statements therefore are all the more surprising since a large part of the cases here reproduced have already been described in the literature. Just this activatibility of normal amboceptors

<sup>1</sup> Erdlich and Morgenroth, page 11; Neisser and Döring, *Berl. klin. Wochenschr.* 1901, No. 22; H. Buchner, *Berl. klin. Wochenschr.* 1901, No. 33, H. Sachs, page 181.

by means of serum corresponding to the blood cells employed has very recently been employed by Buchner<sup>1</sup> *exclusively* as a reaction for the presence of normal amoebocytes.

Although the principle advanced by Gruber as an invariable means of differentiation has failed, we are far from identifying normal and specific amoebocytes. As already stated, we believe that in the sense above described it has been proved that they vary. Here we should like to emphasize that, despite individual multiplicity, all amoebocytes belong essentially to a common class of similarly reacting substances.

To us these observations appear of interest also in another direction. Baumgarten<sup>2</sup> ascribes the hemolysis in a foreign serum entirely to the influence of the amoebocytes, which he identifies with the agglutinins. He says that "while in themselves incapable of effecting hemolysis, they put the red blood cells into such a condition that they allow their hemoglobin to escape even on relatively slight osmotic disturbances." Just these slight osmotic disturbances, according to Baumgarten, are caused by the foreign sera whose osmotic tension is changed by heating (inactivation). Hence Baumgarten regards the assumption of complements as entirely unnecessary. In opposition to this we would like to call to mind the numerous combinations described by us (even Bordet has described such for the hemolysins obtained by immunization), in which the blood cells dissolve in their own serum, i.e. in the ideal isotonic medium, if they have previously been treated with an inactive serum (amoebocyte) of a different species. Such cases clearly indicate that hemolysis by means of blood serum has nothing to do with isotonic conditions; that it is rather due to a poisonous action which depends on the reaction of two components—amoebocyte and complement.

## II. Concerning the Variability of the Complements.

The plurality of the complements contained in a serum has been proved by the most varied experiments. A separation of the individual complements of the serum has been undertaken in various sera by means of chemical or thermic influences,<sup>3</sup> by binding with

<sup>1</sup> Buchner, Berl. klin. Wochenschr., 1901, No. 33.

<sup>2</sup> Baumgarten, *ibid.*, No. 50.

<sup>3</sup> Ehrlich and Morgenroth, see pages 11 et seq.; Ehrlich and Sachs, pages 195 et seq.; Wendelstadt, Centralslatt f. Bact., 1902, Vol. 31, No. 11.

blood-cells loaded with amboceptors,<sup>1</sup> by filtration through porous filters,<sup>2</sup> and by the action of a partial anticomplement.<sup>3</sup> But it does not in all cases require even these methods of separation; all that is necessary is a thorough and continued study of the constituents of the native serum of a given species. Variations can thus be observed therein which lead at once to the view of a plurality of complements.

After several years' observation we found horse serum to be of especial interest in this respect, and we shall therefore briefly discuss the complements of this serum.

Horse serum is particularly well adapted for complementing experiments, because, as a rule, it exerts but slight haemolytic effect by itself. Sheep blood, ox blood, goose blood, and others, so far as we know, are not dissolved at all by horse serum, while so far as guinea-pig blood and rabbit blood are concerned there is an extraordinary amount of variation, some horse sera exerting considerable haemolytic effect on one or both of these blood species, others having no effect whatsoever. In this respect not only did the sera of different horses behave quite differently, but we also observed marked chronological variations in the serum of one and the same normal horse. These show how much the haemolytic properties of an individual's serum can vary. The behavior of the serum (always examined in the fresh condition) on the different days is seen in the following table:

TABLE III.

Date.	Amount of Serum.	Haemolysis of	
		Rabbit Blood (5% 1.0).	Guinea pig Blood (5% 1.0).
June 19. ....	2.0	very little	0
	1.5	trace	0
	0.5	0	0
June 22. ....	2.0	trace	complete
	1.5	minimal	"
	1.0	"	little
	0.5	0	"
July 15. ....	2.0	complete	0
	0.6	"	0
	0.3	strong	0

<sup>1</sup> Ehrlich and Sachs, l. c.

<sup>2</sup> Ehrlich and Morgenroth, page 56; E. Neisser and Döring, Berl. klin. Wochenschr. 1901, No. 22.

<sup>3</sup> Marshall and Morgenroth, pages 222 et seq.

Hence within three days the serum of the horse has become strongly hæmolytic for guinea-pig blood without altering its hæmolytic property for rabbit blood, whereas within a further three weeks its properties have almost become reversed, since now it does not dissolve guinea-pig blood at all, and dissolves rabbit blood (which at first was but slightly affected) very strongly. It is worthy of note that in almost every horse serum which we examined for the purpose we found a considerable amount of amboceptor for guinea-pig blood. This amboceptor was characterized by a particularly high degree of thermolability, being invariably destroyed by heating to 55° C. A complement for the same is very often absent, and even when present it is only on the addition of considerable amounts of fresh guinea-pig serum that this amboceptor becomes manifest.

The cause of this varying hæmolytic property of the horse serum, which is in contrast to the extraordinarily constant amount of normal hæmolysin present in other sera, e.g. goat serum and dog serum, is perhaps due in part to the unusual lability of the complements here concerned. We often observed that a horse serum which dissolved guinea-pig or rabbit blood completely lost this property, or nearly so, by keeping the serum on ice for twenty-four hours, a behavior which we never met with in other sera.

In a similar manner horse serum shows its variability when it is employed purely as a source of complement. We have frequently used horse serum as complement in the following combinations:

Number.	Blood.	Amboceptor.
1	guinea-pig	goat serum
2	rabbit	dog serum
3	"	ox serum
4	guinea-pig	goat serum
5	"	dog serum
6	"	ox serum
7	sheep	dog serum
8	"	serum of a goat immunized with sheep blood

Of all these cases only the complement for 6 and for 8 was present in considerable amounts. So far as the other six complements were concerned we observed a fundamental difference between the experiments which we had made some years ago in Steglitz and those made during the past two years in Frankfurt. Whereas formerly



all of the completions of normal amboceptors succeeded, we found in Frankfurt that we obtained negative results in the great majority of the experiments. The complements necessary for the completion of almost all normal amboceptors were absent, while complements were present for a certain normal amboceptor (guinea-pig blood, ox serum), and for one obtained by immunizing a goat with sheep blood.<sup>1</sup>

This behavior indicates clearly enough a plurality of the complements in a serum, and we do not doubt that further investigations will show the same to be true for the partial complements of other sera. The occasional absence of one or the other complement will most easily be discovered just in the completion of normal amboceptors, for here but few amboceptors have to be considered. Of the numerous amboceptors produced by immunization in many cases, at least a few will find fitting dominant complements. According to our observations, conclusions can be drawn only with the greatest care from isolated negative completion experiments. One cannot conclude that an amboceptor is absent from the impossibility to reactivate normal inactive sera by means of several other active sera.

For the evaluation of *bactericidal* sera in animal experiments we believe it to be especially important to consider cases of this kind. The entire absence or a marked diminution of complements<sup>2</sup> which functionate as dominant complements for certain bactericidal amboceptors may lead to a disturbance in the regularity of a series of experiments, disturbances which show themselves in the fact that now and then an animal dies of the infection even though in the zone of sufficient immune serum to protect the animal. Such irregularities are quite common in the usual test series and manifest themselves frequently in the evaluation of bactericidal sera, where they then are very disturbing.

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<sup>1</sup> In respect to its complements horse serum occupies a special place among most other sera used in the laboratory. Thus, for example, we were rarely successful in complementing the amboceptor of a rabbit immunized with ox blood; we never found a complement in horse sera for the amboceptors of geese or goats immunized with ox blood. That the locality plays a certain rôle in these phenomena follows from our observations that *here*, in contrast to the statements of so reliable an observer as P. Müller in *Graz*, rabbit blood is not dissolved by duck serum to any appreciable extent.

<sup>2</sup> Another abnormal phenomenon which is often observed in this connection, the disturbing action of large amounts of the immune serum, is explained by the peculiar deflection of complements by an excess of amboceptor, as has been determined by M. Neisser and Wechsberg (see pages 120 et seq.).

It is hardly to be doubted that such variations of the complement are responsible for the occasional failures of bactericidal sera in practice, especially if we consider that in diseased conditions a marked diminution or a disappearance of the complements can take place (Ehrlich and Morgenroth, Metchnikoff, Wassermann, Schütze and Scheller).

## XXIII. THE PRODUCTION OF HÆMOLYTIC AMBOCEPTORS BY MEANS OF SERUM INJECTIONS.<sup>1</sup>

### A Contribution to Our Knowledge of Receptors.

By J. MORGENROTH, Member of the Institute.

As a result of the side-chain theory of immunity, and especially in consequence of the conception of "receptor" which this theory brings with it, our views concerning the cytotoxins have to a great extent been emancipated from the *morphological* point of view and placed on a *chemical* basis. This is seen most clearly by looking at the complex hæmolysins of serum, for of all the various cytotoxins these have been most clearly analyzed.

As is well known, if an animal is injected with erythrocytes of a foreign species, there develop in the serum of this animal new substances, the *hæmolytic amboceptors* (immune bodies). The amboceptors are bound, above all, by the red blood-cells of that species whose blood was used for the injection, and it is through this binding that the amboceptors make possible the hæmolytic action of the complement contained in fresh serum. According to the side-chain theory the anchoring of the amboceptors is the result of chemical processes, which again are based on the existence of certain groups of the blood-cells' protoplasm, the *receptors*. If on the basis of this theory one has once clearly seen that the specific binding is strictly a chemical reaction between receptor and amboceptor (or rather between their haptophore groups), it becomes quite evident that the morphological structure of the cell concerned in the reaction is something quite secondary. This is, of course, apart from certain practical points which are mainly the indicators of the deleterious action exerted by the coaction of amboceptor and complement. Among these would be, in this case, escape of hæmoglobin; in the cases of other cytotoxins, disintegration and solution of the cell, cessation

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<sup>1</sup> Reprint from the Münch. med. Wochenschr. 1902, No. 25.

of the motion of flagella and cilia. The specific binding of the amboceptors is therefore not dependent on a coarser or finer morphological structure: *it can occur wherever the specifically related receptors are present.*

For the doctrine of immunity these views constitute a new and really concise definition of specificity. The latter thus loses the systematic character originally given it by botany and zoology and must from now on be regarded purely chemically, as absolutely dependent on the conceptions as to the nature of the cell's receptors. *Every product of immunization is specific for those receptors by which it was called forth, irrespective of where the receptors may be.*<sup>1</sup> When injected into an animal the receptor produces antibodies, and these again, when they meet the receptor under suitable conditions, are bound by the receptor. This binding, in our conception, always remains specific. It matters not whether the receptor is peculiar to the protoplasm of that species of cell which originally excited the immunity, or whether it belongs to a different kind of cell of the same species or to one of a strange species.

Hence the principle of specificity of the amboceptors produced by immunization is not violated when, for example, v. Dungern obtains hæmolytic amboceptors by injections of ciliated epithelial débris, such as is contained in goat milk. v. Dungern<sup>2</sup> has very properly pointed out this fact in emphasizing the community of the receptors. The same holds true for the hæmolytic amboceptors obtained by Moxter<sup>3</sup> by injections of spermatozoa. Several different zoological species, such as goat, sheep, and ox, possess a number of common receptors in their blood-cells.<sup>4</sup>

On the basis of the side-chain theory as it has just been laid down it is almost a matter of course that these receptors of the protoplasm which excite the production of the amboceptors are normally present *dissolved* in the body fluids, a physiological prototype of what occurs to such a high degree in consequence of immunization.<sup>5</sup>

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<sup>1</sup> See the explanations by Ehrlich concerning the receptor apparatus of the red blood-cells in *Schlussbetrachtungen*, Vol. VIII, of *Nothnagels spezielle Pathol. und Therapie*, Vienna, 1901.

<sup>2</sup> v. Dungern, *Münch. med. Wochenschr.* 1899, No. 38.

<sup>3</sup> Moxter, *Deutsche med. Wochenschr.* 1900, No. 1.

<sup>4</sup> Ehrlich and Morgenroth, page 88.

<sup>5</sup> It has already been shown that as a result of injection of amboceptors into sensitive animals a considerable number of cell receptors are thrust off, which

The extraordinary multiplicity of such dissolved substances in blood serum has already been pointed out by Ehrlich.<sup>1</sup> "The chief tools of the internal metabolism are the receptors of the first, second, and third order. They are constantly being used up and produced anew, and can readily therefore, when overproduced, get into the circulation. Considering the large number of organs and the complexity of the protoplasm's chemistry it need not be surprising if the blood, the representative of all the tissues, is filled with an infinite number of the most diverse receptors. Of these we have thus far learned to distinguish the various kinds of lysins, agglutinins, coagulins, complements, ferments, antitoxins, anticomplements, and anti-ferments."

These free receptors when injected into a suitable foreign animal species should therefore show their identity with those of the cells by the fact that, like the latter, they produce immune bodies identical with those produced in the usual way.

A few isolated observations have been made in this direction, but the conclusions following therefrom according to the theory have not been drawn. Thus v. Dungern<sup>2</sup> has observed the development of a hæmolysin directed against chicken erythrocytes as a result of injections of chicken serum into guinea-pig serum, and Tschistovitsch<sup>3</sup> has observed the formation of a hæmolysin (besides agglutinins) on injecting rabbits with horse serum.<sup>4</sup>

For some time past I have made experiments of this kind to demonstrate the existence in goat serum of free receptors identical with receptors of goat erythrocytes. These studies were prompted by the observation that a few normal goat sera exerted a slight inhibiting action on the amboceptors of rabbits immunized with ox blood, an action which Ehrlich and Morgenroth had shown to be due to an anti-immune body.<sup>5</sup> I am led to publish these experiments now

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then functionate as anti-immune bodies. See Ehrlich and Morgenroth, pages 23 and 88.

<sup>1</sup> Ehrlich, *Schlussbetrachtungen*, l. c.

<sup>2</sup> v. Dungern, *Münch. med. Wochenschr.* 1899.

<sup>3</sup> Tschistovitsch, *Annal. Inst. Pasteur*, 1899.

<sup>4</sup> The increase in hæmolytic action of rabbit serum for chicken blood after the injection of chicken blood-plasma, described by Nolf (*Annal. Inst. Pasteur*, 1901), rests apparently only on an increase of complement, not on the development of new amboceptors.

<sup>5</sup> See pages 88 et seq.

because of a rather important contradiction which exists between them and certain experiments recently published by Schattenfroh.<sup>1</sup> This author found that one can produce *hæmolytic* immune bodies for goat blood by injecting rabbits with *goat urine*. He was unable, however, to obtain these immune bodies by injection of the corresponding serum. It must at once be regarded as extraordinary that immune bodies which evidently are excreted through the kidney regularly and plentifully should be absent from the serum itself. It would, of course, have been possible to say that the concentration of the receptors in the serum was small compared to that in the urine, as is the case, for example, with urea, uric acid, and other substances. But the casual antiamboceptor action of the serum prevented this, and pointed to the presence in this of the dissolved receptors. As a matter of fact, therefore, the "interesting contradiction" described by Schattenfroh as existing between the action of the urine and the serum does not obtain; for it is possible by injecting rabbits with goat serum completely deprived of blood-cells to produce specific amboceptors. These amboceptors, to be sure, do not become manifest if the usual methods of investigation, such as have been employed by Schattenfroh, are followed. They are, however, readily and surely demonstrated if one attends to certain fine details.

As a rule a hæmolytic serum obtained by specific immunization will, when fresh, dissolve the corresponding blood-cells; for, as v. Dungern has shown, in immunization with blood-cells the complements usually do not in any sense suffer a change. Only *one* exception is thus far known in this respect, namely, the injection of goat serum into the organism of a rabbit. Ehrlich and Moregnroth<sup>2</sup> have shown that the injection of goat serum into rabbits is followed by the loss of certain complements of the rabbit serum, a loss which is caused by the development of anticomplements directed against the complements of their own serum. These anticomplements are therefore to be regarded as auto-anticomplements. They not only suffice to neutralize the complements present in the serum, but are able to bind complement subsequently added. Thus the amboceptor of a rabbit mixed with goat serum is completely obscured; for if the immune serum is employed fresh, the fitting complements enabling it to act are lacking, while if the serum is inactivated and one seeks

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<sup>1</sup> Münch. med. Wochenschr. 1901, No. 31.

<sup>2</sup> See pages 71 et seq.

to activate it by the addition of normal rabbit serum, the complements of the latter will be made inert by the auto-anticomplement present. Since these auto-anticomplements, however, have no influence on the binding of the amboceptor, the rational mode of procedure is at once indicated. The blood-cells are mixed with the serum of the immunized rabbits and the mixture allowed to stand until the amboceptors present have been bound by the blood-cells. The latter are then separated by centrifuge, the supernatant fluid which contains the cause of the trouble, the auto-anticomplement, being removed. If the blood-cells are now mixed with fresh normal rabbit serum, the hæmolysis which ensues in the incubator will show the presence of the anchored amboceptor. Should this method, which guards against all errors, prove successful, one can also get round the difficulty in an easier manner by using guinea-pig serum as complement. Against this serum, according to our experience, the auto-anticomplement is ineffective. This method, however, does not suffice if we wish to obtain results which permit of only one interpretation. In order surely to avoid another source of error it is well to modify the test still further.

It has been found that normal rabbit serum possesses a considerable though variable hæmolytic action for goat blood (see Table I). The question whether we are dealing with an amboceptor artificially produced or with one which was originally present requires detailed preliminary examination and control tests, and even then is very uncertain because the amboceptor normally present finds a supply of complement in guinea-pig serum more plentiful even than that in rabbit serum itself, as can be seen on reference to the table. This difficulty is avoided without further trouble if the amboceptors produced by immunization and which it is desired to find are taken out of the fluid by means of ox blood-cells instead of goat blood-cells. Because of the partial community of receptor of these two blood-cells this is perfectly allowable. As a rule, too, normal rabbit serum dissolves ox blood only very little, even though considerable complement is present. (See Table I.)

The experiments from which the conclusions are drawn in this study were therefore always made with ox blood. One cc. of a 5% suspension of ox blood-cells is mixed with varying amounts of serum from a rabbit immunized with goat serum, the mixture kept at 38° C. on a water-bath for one hour, then centrifuged, and either fresh rabbit serum added after the supernatant fluid had been decanted, or acti-

TABLE I.

HÆMOLYSIS OF GOAT BLOOD (1 cc. 5%) BY FRESH SERUM OF NORMAL RABBITS.

Rabbit Serum.	I.	II.	III.	IV.	V.	VI.	VII.
0.25	strong	moderate	little	moderate	complete	little	fair
0.1	moderate	little	0	very little	—	0	0
0.05	very little	trace	0	0	very little	0	0

HÆMOLYSIS OF GOAT BLOOD BY THE SAME RABBIT SERA ACTIVATED WITH 0.15 GUINEA-PIG SERUM.

0.25	complete	complete	complete	complete	complete	complete	complete
0.1	"	"	strong	almost	"	strong	"
0.075	"	"	—	complete	"	—	strong
0.05	"	almost	—	strong	"	—	—
0.025	—	complete	—	—	"	—	—

HÆMOLYSIS OF OX BLOOD BY THE SAME RABBIT SERA ACTIVATED WITH 0.15 GUINEA-PIG SERUM.

0.5	trace	faint trace	faint trace	faint trace	trace	very little	fair
0.25	0	0	0	0	faint trace	trace	moderate
0.1	0	0	0	0	0	0	little

The *fresh* rabbit sera, even in amounts of 0.5, do not by themselves exert any hæmolytic effect on ox blood.

vation was effected by the addition of normal guinea-pig serum. The hæmolytic action of the immune sera is seen in Table II.

Rabbits were treated with goat serum which had been carefully freed from all blood-cells by continued centrifuging. Usually the serum was inactivated by heating it to 55° C. for half an hour, then it was injected intraperitoneally. As a rule the animals received two to three injections of increasing doses of serum, in all about 35–90 cc. More frequent injections caused no greater formation of amboceptors, a behavior which corresponds to that seen with the injection of ox blood or goat blood.

These experiments show that specific amboceptors were developed in all the rabbits treated with goat serum. Quantitatively this was subject to individual fluctuations just as is seen following the injection of blood-cells; in some cases the development was quite considerable. Most of the sera were examined fresh for their action on ox blood, and invariably showed themselves without action even in doses of 0.5 cc.<sup>1</sup> The addition of large amounts of normal rabbit

<sup>1</sup> The method here employed to disclose amboceptors whose presence is masked can often be used with success. Dr. Marshall and I shall shortly report an analogous case dealing with the amboceptors of a pathological exudate.



TABLE II.

1.0 cc. 5% Ox Blood.

A. Blood+amboceptor are kept at 37° C. for one hour. After centrifuging the fluid is decanted and the sediment mixed with 2 cc. physiological salt solution and 0.2 cc. rabbit serum as complement.

		Complete Hæmolysis.
Serum rabbit	I .....	0.05 cc.
" "	II .....	0.05 "
" "	III.....	0.25 "

B. BLOOD+AMBOCEPTOR+0.1-0.2 GUINEA-PIG SERUM AS COMPLEMENT.

Serum rabbit	IV.....	0.1 cc.
" "	V.....	0.05 "
" "	VI.....	0.05 "
" "	VII.....	0.028 "
" "	VIII.....	0.013 "
" "	IX.....	more than 0.25 "
" "	X.....	0.05 "
" "	XI.....	less than 0.05 "

serum does not suffice to overcompensate the auto-anticomplement present. For example, the serum of rabbit III shows the following solvent action after the addition of 0.6 cc. rabbit serum:

0.5 cc.....	0	0.075 cc. ....	very little
0.25 " .....	trace	0.05 " .....	" "
0.15 " .....	"	0.025 " .....	trace
0.1 " .....	very little		

The abnormal course of this slight hæmolysis shows very well the interference of anticomplement on the one hand and of the amboceptor on the other.

The similarity of the amboceptor produced by injections of goat serum to that produced by injections of blood is more plainly seen by the fact that the anti-immune body obtained by immunization acts against the former amboceptor just as well as against the latter. Table III shows this behavior very well.

The anti-immune body used was contained in the inactivated serum of a goat which had been injected several times with the serum of rabbits immunized with ox blood. 0.3 cc. of this anti-immune body serum were mixed with varying amounts of the amboceptor sera to be tested and the mixtures kept at room temperature for one hour. Thereupon 1 cc. of a 5% suspension of ox blood-cells was added to

each specimen, which was then kept on a water-bath at 38° C. for one hour, after which the mixtures were centrifuged. The blood-cell sediment was again suspended in salt solution and 0.15 cc. guinea-pig serum added as complement. The solution which then ensued was a measure for the bound amboceptor, or for the deflection by the antiamboceptor. Control tests were made with 0.3 cc. normal inactive goat serum in parallel experiments.

TABLE III.

A. INHIBITION OF THE AMBOCEPTOR OF THE RABBIT  
TREATED WITH GOAT SERUM.

Amount of Amboceptor.	+0.3 Antiamboceptor.	+0.3 Normal Inactive Goat Serum.
0.25	complete solution	complete solution
0.15	strong	" "
0.1	little	" "
0.075	very little	" "
0.05	0	" "
0.025	0	strong

B. INHIBITION OF THE AMBOCEPTOR OF THE RABBIT  
TREATED WITH GOAT BLOOD.

0.2	complete solution	complete solution
0.15	strong	" "
0.1	little	" "
0.075	trace	" "
0.06	0	" "
0.05	0	moderate
0.025	0	little
0.012	0	trace
0.009	0	0

The antiamboceptor is thus seen to offer exactly the same protection against the amboceptors obtained as a result of *goat-blood* injections and those resulting from *goat-serum* injections, whereby their identity is demonstrated.

The presence of free receptors in the urine and serum leads to the conclusion that an active receptor metabolism exists in the organism of the goat; in other words, that receptors are constantly reaching the serum from the cells and are then excreted by the kidney. Whether one is here dealing with decomposition products or with the products of some secretion or other cannot be determined. The

fact that free receptors leave the serum to reappear in the urine seems to make it probable that they have no significance for the organism itself. On the contrary, one may suspect that these are products of regressive metabolism which are eliminated from the body as useless. The explanation that the free receptors originate from the breaking down of red blood-cells or other cells is entirely sufficient. It may be, however, that there is a physiological thrusting-off of the same which bears some relation to their nutritive function. In view of the elimination through the urine, it seems improbable that this constitutes a regular function as anti-immune body against the action of a possible autolysin. That certainly would be an unsuitable process. In fact the free receptors evidently do not generally possess the character of antiautolysins, as Besredka<sup>1</sup> believes, for by injecting a rabbit with ox serum it was impossible to obtain any hæmolytic amboceptors. This corresponds to the negative results obtained by London<sup>2</sup> on injecting guinea-pigs with rabbit serum.

One thing is clearly shown by the presence of *dissolved* substances capable of producing amboceptors, namely, that without the idea of "receptors" a universally applicable conception of the origin and mode of action of the cytotoxins is impossible, as is also a clear conception of the nature of "specificity."

*Subsequent Note.*—In a recently published study (Münch. med. Wochenschr. 1902, No. 32) P. Th. Müller reports on the production of hæmolytic amboceptors by treating pigeons with guinea-pig serum, and he accepts the views here developed.

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<sup>1</sup> Besredka, Annal. de l'Institut Pasteur, Oct. 1901.

<sup>2</sup> London, Arch. des Sciences biologiques, St. Petersburg.

## XXIV. THE QUANTITATIVE RELATIONS BETWEEN AMBOCEPTOR, COMPLEMENT, AND ANTICOMPLEMENT.<sup>1</sup>

By Dr. J. MORGENROTH, Member of the Institute, and Dr. H. SACHS, Assistant at the Institute.

### I. Amounts of Amboceptor and Complement Required.

EVERY laboratory in which systematic quantitative studies are made on hæmolysis will have had encountered the relations existing in different cases between the amounts of amboceptor and complement necessary for hæmolysis. Attention was first called to these relations by v. Dungern,<sup>2</sup> who described a hæmolytic experiment with ox blood + amboceptor from a rabbit immunized with ox blood + rabbit serum as complement. In this case he noticed that in order to accurately find the minimal amount of a completing serum necessary for hæmolysis, it was necessary to employ a high multiple of that amount of amboceptor which is sufficient to effect complete solution when a large excess of complement is present. In determining the amount of complement required, v. Dungern therefore employed sixteen times the required amount of amboceptor. Gruber also says recently that "highly prepared (sensitized) human blood-cells," in consequence of their preparatory treatment, are dissolved by a minimum of active normal serum.

In the following we wish to describe several interesting observations made by us in the course of several years.

We shall begin by describing a number of different cases in which the relations between the amount of amboceptor necessary for complete solution and that of the completing serum were studied. In the experiments 1 cc. of a 5% suspension of the blood-cells is always used. Especial emphasis is laid on the fact that in the comparative tests all the test-tubes contained the same volume of fluid.

The first experiments were made with sheep blood + amboceptor of a goat immunized with sheep blood + guinea-pig serum as complement. (See Table I.)

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<sup>1</sup> Reprint from the Berl. klin. Wochenschr. 1902, No. 35.    <sup>2</sup> See page 38.  
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TABLE I.

1 CC. 5% SHEEP BLOOD+AMBOCEPTOR OF GOATS TREATED WITH SHEEP  
BLOOD+GUINEA-PIG SERUM AS COMPLEMENT.

Amount of Amboceptor.	Proportion of the Amounts of Amboceptor.	Amount of Complement Sufficient for Complete Solution.	Proportion of the Amounts of Complement.
I.			
0.05	1×	0.008	$\frac{1}{3.2}$
0.2	4×	0.0025	$\frac{1}{5.6}$
0.4	8×	0.0014	$\frac{1}{5.6}$
II.			
0.025	1×	0.04	$\frac{1}{1.6}$
0.038	1.5×	0.025	$\frac{1}{1.6}$
0.05	2×	0.025	$\frac{1}{2}$
0.075	3×	0.02	$\frac{1}{2.5}$
0.1	4×	0.016	$\frac{1}{4}$
0.2	8×	0.01	$\frac{1}{10}$
0.5	20×	0.004	$\frac{1}{10}$
III.			
0.05	1×	0.1	$\frac{1}{3.3}$
0.1	2×	0.03	$\frac{1}{10}$
0.2	4×	0.01	$\frac{1}{10}$
0.4	8×	0.01	$\frac{1}{10}$
IV.			
0.05	1×	0.08	$\frac{1}{5.3}$
0.1	2×	0.015	$\frac{1}{20}$
0.2	4×	0.004	$\frac{1}{20}$

The figures in Table I show that in the four similar cases here examined the relation between the amount of amboceptor and of the complement required is such that *in the presence of larger amounts of amboceptor smaller doses of complement suffice for complete haemolysis*. The relation is not exactly the same in the separate cases, as can readily be seen from the figures of columns 2 and 4. In one case (I) increasing the amboceptor eight times reduced the amount of complement required only to  $\frac{1}{5.6}$ , whereas in another case (IV) increasing the amount of amboceptor only four times reduced the complement required to  $\frac{1}{20}$ . This shows us at once that there is no definite ratio between the two factors. The causes of this varying relation will be discussed later.

The phenomenon in question is much less marked in the cases reproduced in Table II, in which the combination was ox blood + the amboceptor of specifically immunized rabbits + guinea-pig serum or rabbit serum as complement.

TABLE II.

A. 1 CC. 5% OX BLOOD + AMBOCEPTOR OF RABBITS TREATED WITH OX BLOOD + GUINEA-PIG SERUM AS COMPLEMENT.

Amount of Amboceptor.	Proportion of the Amounts of Amboceptor.	Amount of Complement Sufficient for Complete Solution.	Proportion of the Amounts of Complement.
0.002	1×	0.035	1
0.005	2½×	0.015	$\frac{1}{2.3}$
0.01	5×	0.01	$\frac{1}{3.5}$
0.05	25×	0.008	$\frac{1}{4.4}$
0.1	50×	0.008	$\frac{1}{4.4}$
0.2	100×	0.008	$\frac{1}{4.4}$
0.4	400×	0.01	$\frac{1}{3.5}$

TABLE II—*Continued.*

## B. THE SAME, BUT RABBIT SERUM AS COMPLEMENT.

Amount of Amboceptor.	Proportion of the Amounts of Amboceptor.	Amount of Complement Sufficient for Complete Solution.	Proportion of the Amounts of Complement.
I.			
0.005	1×	0.5	$\frac{1}{1}$
0.01	2×	0.17	$\frac{1}{2.9}$
0.05	10×	0.12	$\frac{1}{4.2}$
0.1	20×	0.14	$\frac{1}{3.6}$
0.2	40×	0.14	$\frac{1}{3.6}$
0.4	80×	0.15	$\frac{1}{3.3}$
II.			
0.005	1×	0.6	$\frac{1}{1}$
0.01	2×	0.17	$\frac{1}{2.5}$
0.05	10×	0.12	$\frac{1}{5}$
0.1	20×	0.14	$\frac{1}{4.3}$
0.2	40×	0.14	$\frac{1}{4.3}$
0.4	80×	0.15	$\frac{1}{4}$
III.			
0.005	1×	0.75	$\frac{1}{1}$
0.0075	$1\frac{1}{2}$ ×	0.6	$\frac{1}{1.25}$
0.015	3×	0.14	$\frac{1}{5.3}$
0.03	6×	0.17	$\frac{1}{4.4}$
0.06	12×	0.14	$\frac{1}{5.3}$
0.12	24×	0.12	$\frac{1}{6.3}$

Here we see that the employment even of very high multiples of the amboceptor effects a reduction in the amount of complement required of one-third to one-sixth at the most. But what is particularly characteristic for this case is the fact that the minimal amount of complement is almost reached with a small multiple of the "amboceptor unit,"<sup>1</sup> and that it does not materially change with a further increase of the amboceptor. Thus, in Table II, A, we see that when five times the amboceptor unit is employed the amount of complement required is 0.01; when 25, 50, or 100 times the unit is employed the complement is 0.008. Table II, B, shows that with the employment of two to three times the amboceptor unit the maximum of complement action is already attained.

An entirely analogous behavior is shown by the cases in Table III, in which the same blood and the same amboceptor are used as in Table I, but in which different kinds of complement are added, namely, sheep serum and horse serum.

These cases constitute the transition to those reproduced in Table IV which deal with ox blood + the amboceptor of goats treated with ox blood + three different complements, namely, guinea-pig, rabbit, and sheep serum respectively. *In these also a limit is reached beyond which the decrease of complement required is but slightly or not at all affected by an increase in the amount of amboceptor.*

We see therefore that *with an increase of the amount of amboceptor the amount of complement required at one time drops to a greater or less degree, at another time it remains unchanged.* Upon what does this phenomenon depend? In order to explain this we must consider three factors which may be combined with one another, and which must be considered in each individual case. These are: 1. The receptors present in the red blood-cell. 2. The conditions of affinity. 3. The plurality of the amboceptors.

So far as the first point is concerned we know that the amount of receptors of the red blood-cells may exhibit great differences in any individual case.<sup>2</sup>

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<sup>1</sup> We use the term "amboceptor unit" to specify that amount of amboceptor which on the addition of the optimal amount of complement just suffices for complete hemolysis. In the same sense R. Pfeiffer uses the term "immunity unit" when speaking of bactericidal sera. Corresponding to the amboceptor unit the "receptor unit" is that amount of receptor which binds the amboceptor unit.

<sup>2</sup> See Ehrlich, *Schlussbetrachtungen in Nothnagels spec. Pathologie und Therapie*, Vol. VIII, Vienna, Hölder, 1901; and Ehrlich and Morgenroth, page 71.



TABLE III.

A. 1 cc. 5% SHEEP BLOOD+AMBOCEPTOR OF GOATS TREATED WITH SHEEP BLOOD+SHEEP SERUM AS COMPLEMENT.

B. THE SAME, BUT WITH HORSE SERUM AS COMPLEMENT.

Amount of the Amboceptor.	Proportion of the Amount of Amboceptor.	Amount of Complement which Suffices for Complete Solution.	Proportion of the Amounts of Complement.
A.			
0.1	1 ×	0.15	1
0.25	2.5 ×	0.035	$\frac{1}{4.3}$
0.5	5 ×	0.05	$\frac{1}{3}$
0.75	7.5 ×	0.05-0.035	$\frac{1}{3}$ to $\frac{1}{4.3}$
B.			
0.1	1 ×	0.5 almost [complete]	1
0.2	2 ×	0.1	$\frac{1}{5}$
0.4	4 ×	0.1	$\frac{1}{5}$
0.8	8 ×	0.1	$\frac{1}{5}$

One erythrocyte may possess just so many receptors for a certain poison as are necessary to bind a single solvent dose, i.e. there is present just a receptor unit, whereas in other cases such a multiple of the receptor unit may be present that a hundred times the amboceptor unit is bound. In bacteria the latter condition is present to a still very much greater degree: agglutinins (Eisenberg and Volk) and bacteriolytic amboceptors (R. Pfeiffer) are bound in enormous excess, frequently as high as many thousand times the effective amount. It is therefore entirely clear that these conditions must exercise a deciding influence on the fact whether an increased amount of immune serum decreases the amount of complement required or not. It may be regarded as self-evident that in all those cases in which only the single effective dose can be bound, i.e. in which only *one* amboceptor unit is anchored, an excess of amboceptor can never exert a favorable influence; on the contrary an increase in the

amount of complement can readily result owing to the deflection phenomenon whose significance was first pointed out by M. Neisser and Wechsberg.<sup>1</sup>

TABLE IV.

A. 1 cc. 5% Ox BLOOD + AMBOCEPTOR OF GOATS TREATED WITH OX BLOOD + GUINEA-PIG SERUM AS COMPLEMENT.

B. THE SAME + RABBIT SERUM AS COMPLEMENT.

C. THE SAME + SHEEP SERUM AS COMPLEMENT.

Amount of the Amboceptor.	Proportion of the Amounts of Amboceptor.	Amount of Complement which Suffices for Complete Solution.	Proportion of the Amounts of Complement.
A.			
0.1	1×	0.01	1
0.2	2×	0.01	1
0.4	4×	0.01	1
0.8	8×	0.01	1
B.			
0.1	1×	0.15	1
0.2	2×	0.15	1
0.4	4×	0.15	1
0.8	8×	0.15	1
C.			
0.1	1×	0.1	1
0.2	2×	0.1	1
0.4	4×	0.1	1
0.8	8×	0.075	$\frac{1}{1.4}$

The problem is more difficult in those cases in which the red blood-cells contain a plurality of receptor units and therefore bind a multiple of amboceptor units. In these cases the result of the experiments will depend mainly on the following factors.

We know that as a rule the affinity of the amboceptor's complementophile group is increased when the *cytophile* group is anchored by the receptors. If this relative increase of affinity is very large, the added complement will combine exclusively with the anchored amboceptor, and in certain doses will effect solution. In this case

<sup>1</sup> M. Neisser and Wechsberg, see page 120.

the required equivalence will already be reached with the amount of complement just sufficient for solution, and an increase of the complement action by loading the blood-cells with additional amboceptor will not occur.

The conditions, however, are entirely different if the affinity of the complementophile group of the anchored amboceptor for the complement is only very slight; in other words, when we are dealing with an easily dissociated combination in a reversible process. In that case, in accordance with a well-known chemical law, *the more of one of the elements is in excess, the more of the completed combination will remain intact*. Hence if there are very few receptor units in the blood-cells, it will be necessary to add very much complement in order to diminish the amount of dissociation and to cause the formation of an effective unit of hæmolysin; if more receptor units are present, less complement will suffice. The tables here given present numerous considerations which show that *little amboceptor + much complement* and *much amboceptor + little complement* lead to the formation of the *same* amount of *complement-amboceptor* combination (hæmolysin unit) anchored by the receptors.

A most conspicuous rôle, however, is played by the fact that *the immune serum is not a simple substance, but is made up of partial amboceptors to which various dominant complements of the sera correspond*. Of especial importance in this respect are partial amboceptors present in immune serum in *small* amounts (and which therefore can only come into action when high multiples of the immune serum are employed), but which, for their completion, find a partial complement *which is particularly plentiful* in the completing serum. Such a partial amboceptor present in these small amounts (such, for example, as has been demonstrated in the serum of rabbits treated with ox blood) constitutes one of the main explanations for the phenomena above described.

From these considerations we see that the various phenomena which we observe in the interdependence of the amounts of amboceptor and complement required may have entirely different causes, but that, by regarding all of the three above-mentioned factors, these phenomena can be explained very naturally. Under these circumstances it is, of course, not permissible to generalize from one particular case.

## II. Amount of Amboceptor and Anticomplement Required.

The following observations deal with the quantitative relations existing between the amount of amboceptor and that of the anticomplement required to prevent hæmolysis. In a number of cases we determined the amount of anticomplement which just suffices to prevent the solution of red blood-cells loaded with varying amounts of amboceptor, when that amount of complement was present which always just sufficed for complete solution.

The majority of our experiments again refer to the solution of sheep blood by an immune serum (derived from a goat) whose amboceptor is complemented by guinea-pig serum. This, it will be recalled, is the case in which with large amounts of amboceptor the complement required decreases considerably. For the anticomplement we made use of the serum of a goat which had previously been treated with repeated injections of rabbit serum. This serum, as can be seen from a previous communication, does not only protect against the complement of rabbit serum, but also against those of guinea-pig serum.

To begin, the amount of completing guinea-pig serum was determined which, with varying amounts of amboceptor, sufficed for the complete solution of 1 cc. 5% sheep blood. After this the quantity of anticomplement required in each instance to effect neutralization was determined, whereupon complement and anticomplement were mixed and kept at 37° C. in an incubator for half an hour. Blood and amboceptor were then added. Such an experiment is reproduced in Table V.

As shown in the table by the degree of hæmolysis, the peculiar behavior is observed that with small amounts of amboceptor 0.015 cc. anticomplement serum neutralize the complement of 0.05 in guinea-pig serum, whereas with large amounts of amboceptor 0.35 cc. anticomplement serum are required to neutralize 0.006 guinea-pig serum. If we calculate the amount of complementing serum neutralized in both cases by 1 cc. anticomplement serum, we find that in one case it is 3.3 cc., in the other 0.017 cc. Hence *when large amounts* of amboceptor are employed the anticomplement acts 195 times weaker. The required amount of anticomplement is therefore absolutely dependent on the quantity of the amboceptor employed. This becomes most evident by the fact that even with equal amounts of

complement required, but with varying additions of amboceptor (see columns *a* and *b* of Table V), different amounts of anticomplement (corresponding to the amount of amboceptor present) are required to neutralize the complement, more being required with larger amounts of amboceptor. *In these cases, therefore, the amount of anticomplement required is far from being a simple function of the amount of complement, but is dependent on the amount of amboceptor present.*

TABLE V.

## A.

Amount of the Amboceptor.	Amount of the Complement Sufficient for Complete Solution.
0.3	0.005
0.05	0.005
0.01	0.01
0.005	0.035

## B.

Amount of Anticomplement.	<i>a</i> Amboceptor, 0.3. Complement, 0.006	<i>b</i> Amboceptor, 0.05. Complement, 0.006	<i>c</i> Amboceptor, 0.01. Complement, 0.01.	<i>d</i> Amboceptor, 0.005. Complement, 0.05.
0.35	0	0	0	0
0.25	faint trace	0	0	0
0.15	trace	0	0	0
0.1	"	0	0	0
0.075	moderate	faint trace	0	0
0.05	complete	trace	faint trace	0
0.035	"	moderate	little	0
0.025	"	complete	"	0
0.015	"	"	complete	0
0.01	"	"	"	faint trace
0	"	"	"	complete

In several other combinations, which we analyzed in a similar manner, we met with the same behavior to a greater or less extent. In Table VI such an experiment is reproduced; it deals with the solution of ox blood by an amboceptor derived from rabbits and complemented by guinea-pig serum. As in the previous case, inactive serum of a goat treated with rabbit serum served as anticomplement.

In this case when small amounts of amboceptor are present 1.0 cc. of the anticomplement serum neutralizes 1.0 cc. guinea-pig serum; with larger amounts of amboceptor it neutralizes only 0.067 cc.; i.e., about fifteen times less.

TABLE VI.

OX BLOOD + AMBOCEPTOR OF AN OX-BLOOD RABBIT + GUINEA-PIG SERUM.

Amount of Amboceptor.	Amount of Complement Sufficient to Effect Complete Solution.	
0.2	0.05	
0.004	0.075	
Anticomplement.	Amboceptor, 0.2. Complement, 0.05.	Amboceptor, 0.004. Complement, 0.1.
0.75	0	0
0.5	strong	0
0.35	almost complete	0
0.25	complete	0
0.15	"	0
0.1	"	0
0.075	"	trace
0.05	"	little
0.035	"	moderate
0.025	"	strong
0.015	"	almost complete
0.01	"	complete

The study of the phenomena of immunization has taught us that nothing is so liable to error as premature generalization. Hence we were not at all surprised to find that there are cases in which, in contrast to that above described, the quantity of anticomplement required appeared exclusively to be a function of the amount of complement, and in no way dependent on the degree of occupation of the receptors by amboceptors. Curiously enough this case concerns the combination first described, namely, sheep blood, amboceptor of goats treated with sheep blood, and guinea-pig serum as complement, *with this difference, however, that in this case the anticomplement was not the same, since it was derived from a rabbit treated with guinea-pig serum.* This anticomplement, therefore, so far as its relation to guinea-pig serum was concerned, can be termed "isogenic" in contrast to the anticomplement previously used, which can be termed "alloiogenic," since it was derived by injecting rabbit serum. The experiment is shown in Table VII.

Here we see that neutralization of the ten times larger amount of complement, such as is made necessary by the smaller amount of amboceptor, requires ten times as much anticomplement as it does with one-tenth the quantity of complement when larger amounts of amboceptor are used.

TABLE VII.

Amount of Amboceptor.	Amount of Complement Sufficient for Complete Solution.	Amount of Complement in the Anticomplement Test.	Amount of Anticomplement Required for Complete Neutralization.
0.1	0.02	0.025	0.04
0.2	0.0025	0.0035	0.005

The results of the experiments in the various cases are diametrically opposite, for in one case there is a relation between complement and amount of anticomplement required with different quantities of amboceptor, in other cases there is a wide divergence. How are these phenomena to be explained?

To begin, let us assume for the sake of simplicity that complement and anticomplement are of simple constitution. In that case, if, as all our experiments show, the affinity of complement is much greater for anticomplement than for amboceptor, the neutralization of complement and anticomplement should follow stoichiometric laws. As a matter of fact this is what we found in the last case (Table VII). In the first two cases, however, the results diverge so widely from this, and are moreover so far beyond the limits which might be caused by errors, that from this fact alone it necessarily follows that conditions of affinity cannot by themselves suffice for an explanation. We are therefore compelled to call to our aid another factor, one which we have already emphasized, namely, *the plurality of the complements and anticomplements*.

Let us assume that in this case two dominant complements, *A* and *B*, came into play in the complementing serum. The serum serving as anticomplement must therefore contain the corresponding anticomplement  $\alpha$  or  $\beta$ . It is self-evident that the corresponding anticomplements are present in the *isogenic* serum; that they may also appear in the serum obtained by injection of a different serum, e.g. of rabbit serum, is shown by previous experience. It is not at all necessary to assume that rabbit serum contains exactly the same complements *A* and *B* present in guinea-pig serum; it suffices to assume a *partial* identity for the rabbit serum's complements (*A*<sub>1</sub> and *B*<sub>1</sub>), namely, *an identity in the haptophore group*.

Following the terminology of the theory of numbers in which "friendly numbers" (*numeri amicales*) are spoken of, one could designate complements of different species which correspond in their haptophore groups, as "friendly complements."

Now if one injects any serum containing two different complements, the production of partial anticomplements will to a great extent depend on the relative amount of the two complements. For example, if in one case there is considerable complement *A* and but little *B*, while in another case there is considerable *B* and little *A*, the anticomplement will be directed for the greater part against *A* in the one case, and against *B* in the other. It is therefore readily understood that with isogenic sera the yield of anticomplements can correspond fairly well to the mixture of complements present in the injected material, for the average composition of this mixture is quite constant. A serum thus results which to a certain extent is fitted to the complements of the serum injected.

Since, however, a serum contains, not two complements as we have assumed for the sake of simplicity, but a large number of complements, it can, of course, happen even with isogenic anticomplements that a disharmony will occur so far as certain fractions of complements are concerned. The following case shows that even with an isogenic anticomplement the relative proportion between complement and anticomplement with different amounts of amboceptor is not maintained. (See Table VIII.)

TABLE VIII.

HUMAN BLOOD + AMBOCEPTOR OF A HUMAN-BLOOD RABBIT + RABBIT SERUM +  
ANTICOMPLEMENT FROM THE GOAT TREATED WITH RABBIT SERUM.

Amount of Amboceptor.		Amount of Complement Necessary for Complete Solution.	
0.2		0.05	
0.2		0.05	
0.05		0.075	

Anticomplement.	Amboceptor, 0.2. Complement, 0.05	Amboceptor, 0.1. Complement, 0.05.	Amboceptor, 0.05. Complement, 0.1.
0.1	0	0	0
0.075	0	0	0
0.05	trace	0	0
0.035	"	0	0
0.025	little	trace	0
0.015	moderate	"	trace
0.01	almost complete	little	moderate
0	complete	complete	complete

In this case 1.0 cc. anticomplement neutralizes 4.0 cc. complement when 0.5 cc. amboceptors are present, 1.42 cc. when 0.1 cc. amboceptor is present, and only 0.67 cc. complement with 0.2 cc. amboceptor.



A priori, it is, of course, conceivable that in the rabbit the complements  $A_1$  and  $B_1$  exist exactly in the same proportion as do complements  $A$  and  $B$  in the guinea-pig, but we must admit that this would be a coincidence. In all probability the development of the alloiogenic anticomplement will result in a serum in which the proportion of the two anticomplements is absolutely different, so that, for example, anticomplement  $B$  will be present in much smaller amount than in the isogenic anticomplement serum. The behavior of this will then be as follows: A certain quantity of the isogenic anticomplement serum produced by guinea-pig serum (presupposing that its constitution is uniform) will neutralize guinea-pig serum in such a way that complement  $A$  and complement  $B$  of this mixture are neutralized at the same time. If we proceed to do the same with the alloiogenic anticomplement serum, we find that in the mixture of anticomplement and guinea-pig serum, complement  $A$  is completely neutralized, but that a larger or smaller excess of complement  $B$  is still unsaturated. In those cases in which complement  $A$  is the dominant complement both mixtures will prove neutral; when amboceptors are employed for which  $B$  is the dominant complement, only one of the mixtures will be neutral, the other will still be active.

Now we shall assume that with the employment of large amounts of amboceptor, a partial amboceptor comes into action which is present in the immune serum in relatively small quantity. This partial amboceptor is complemented by complement  $B$  contained in guinea-pig serum, whereas the preponderating amboceptor is sensitized by complement  $A$ . Complement  $B$  finds a plentiful amount of anticomplement in the isogenic immune serum, but not in the alloiogenic serum. In the latter case, therefore, disproportionately much serum containing  $B$  anticomplement will be required in order to inhibit the complement action when large quantities of amboceptor are present. If the difference becomes so great that the anticomplement against complement  $B$  is present only in very slight amounts, we shall have a condition like that described by Marshall and Morgenroth (see page 222). They found an ascitic fluid which was effective only against a particular complement of a serum, while it was entirely inert against another serum of this same species.

We have endeavored to establish this point of view on a wider experimental basis. With this end in view we first used small amounts of amboceptor, adding various multiples of the complementing dose

of serum and then determining the amount of anticomplement required in each case. In one of the experiments we made a parallel test with a large excess of amboceptors. The results showed that under these circumstances, for each of the cases and with a certain amount of amboceptor, the anticomplement required is proportionate to the amount of complement. This is shown in Table IX.

TABLE IX.

1 cc. 5% SHEEP BLOOD+AMBOCEPTOR OF GOATS IMMUNIZED WITH SHEEP BLOOD+GUINEA-PIG SERUM AS COMPLEMENT.

The serum of a goat treated with rabbit serum, as anticomplement.

Amount of Amboceptor.	Amount of Complement.	Amount of Anticomplement Necessary for Complete Neutralization.
<i>A. Little Amboceptor (=1 Amboceptor Unit).</i>		
0.005	0.1	0.22
0.005	0.2	0.4
<i>B. Much Amboceptor (=25 Amboceptor Units).</i>		
0.125	0.006	0.24
0.125	0.012	0.42
0.125	0.024	0.8

1 cc. 5% OX BLOOD+AMBOCEPTOR OF A GOAT IMMUNIZED WITH OX BLOOD+RABBIT SERUM AS COMPLEMENT.

The serum of a goat treated with rabbit serum as anticomplement.

Amount of Amboceptor.	Amount of Complement which is just Fully Neutralized.	Amount of Anticomplement.
0.15*	0.2	0.1
0.15	0.1	0.05
0.15	0.05	0.025

\* =about 2 amboceptor units.

Here, then, we are dealing with the same phenomenon which in the domain of antitoxin immunity we know as the *multiplication of the  $L_0$  dose*. From our standpoint this is easily explained, for if at any point in the saturation of the blood-cells' amboceptors a certain amount of the complement dominant in this case is neutralized by a certain quantity of anticomplement, the other conditions will in no way be altered by a doubling, quadrupling, etc., of the

complement, and the amount of complement and that of anticomplement required remain in the same ratio. A definite relation therefore exists *in every grade of amboceptor saturation* between the amount of complement and that of anticomplement required. This is in contrast to the great differences which appear when the occupation with amboceptors varies. The relation just described indicates that we are here dealing *with a chemical process following stoichiometric laws*.

We should like to mention further that this peculiar behavior observed by us is of some importance in refuting an objection made by Gruber (l. c.) against Wechsberg. As is well known, Gruber believed he had shown that in the bactericidal sera anticomplements were present produced by the immunization. This he held to be very important, since according to his view it showed that the deflection of complements by excess of amboceptors, which had been described by Neisser and Wechsberg, was incorrect. This is not the place to enter into the great improbability of Gruber's deductions, for this has already been well pointed out by Wechsberg, by Lipstein,<sup>1</sup> and by Levaditi.<sup>2</sup> Wechsberg<sup>3</sup> repeated Gruber's experiments, but was unable to confirm his results. Sachs also was unable to do this. Gruber has now objected to Wechsberg's work on the score of a gross error, saying that Wechsberg worked with weakly sensitized blood-cells, whereas he had used strongly sensitized blood-cells. Wechsberg had therefore used considerably more complement than he, and had in consequence required much more anticomplement for neutralization, so that the presence of small quantities of anticomplement could easily have escaped Wechsberg.

*From what has been said above, however, just the contrary occurs; with alloiogenic sera larger amounts of anticomplement are used.* That the anticomplement which would be produced artificially by injections of bacteria (even if that be regarded as conceivable) would eminently be alloiogenic need not further be emphasized. It is shown by Table VIII that the conditions which Gruber assumed to exist do not obtain, even with an isogenic anticomplement, in Gruber's case (human blood+human-blood rabbit+rabbit serum). It is unnecessary to enter further into Gruber's objections, for Wechsberg<sup>4</sup> has succeeded through the demonstration of complementophile amboceptoids in finding the source of the differences. These amboceptoids have meantime been found independently by E. Neisser and Friedemann<sup>5</sup> and by P. Th. Müller.<sup>6</sup>

It is immaterial in judging of this phenomenon whether in the anticomplementary sera used by Gruber the diverting amboceptoids developed as a result of long standing or under the influence of too high an inactivating temperature. The main thing is that even the phenomenon observed by Gruber and used

<sup>1</sup> Lipstein, see pages 132 et seq.

<sup>2</sup> Levaditi, *Compt. rend. Soc. de Biol.* 1902, No. 25.

<sup>3</sup> Wechsberg, *Wiener klin. Wochenschr.* 1902, Nos. 13 and 28.

<sup>4</sup> *Ibid.*

<sup>5</sup> Neisser and Friedemann, *Berl. klin. Wochenschr.* 1902, No. 29.

<sup>6</sup> P. Th. Müller, *Münch. med. Wochenschr.* 1902, No. 32.

by him as an objection constitutes a new and telling demonstration of the correctness of the amboceptor theory.

Thus we see that the anticomplement experiments give us a further insight into the mechanism of hæmolysin action. This in its turn shows that the simple unitarian conception must be abandoned to be replaced by the view maintained by us that the exciting substances as well as the reaction products arising in immunization are exceedingly manifold in character.

## XXV. THE HÆMOLYTIC PROPERTIES OF ORGAN EXTRACTS.<sup>1</sup>

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The first observations concerning the hæmolytic properties of organ extracts were published, so far as we are aware, by Metchnikoff.<sup>2</sup>

Proceeding from his observation that in the peritoneum of the guinea-pig goose blood-cells are taken up by certain phagocytes, the *macrophages*, and digested intracellularly, Metchnikoff sought to demonstrate digestive actions in vitro in extracts of such organs which are rich in macrophages. He regarded the hæmolytic function as an indicator of this digestive action. He found that extracts of certain organs of guinea-pig (but not guinea-pig serum) exerted a hæmolytic action on goose blood; the lymphoid portion of the omentum showed this action quite regularly, the mesenteric glands frequently, and in a limited number of cases the spleen. Of the other organs the pancreas showed a marked, and the salivary glands a weak hæmolytic action; the bone marrow, liver, kidney, brain and spinal cord, ovaries, testicles, and adrenals were inert.

Metchnikoff found the hæmolytic substance to be a soluble ferment contained in the macrophages; he termed it "macrocytase" to distinguish it from the bactericidal ferment derived from microphages, which he calls "microcytase." It shows itself to be a "cytase"<sup>3</sup>

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<sup>1</sup> Reprint from the Berlin. klin. Wochenschr. 1902, No. 37.

<sup>2</sup> Metchnikoff, Annal. de l'Institut. Pasteur, Oct. 1899; see further references in Metchnikoff, l'Immunité, Paris, 1901.

<sup>3</sup> Metchnikoff and his pupils use the term "cytase" for our complements as well as for the complex cytotoxins (hæmolysins, bacteriolysins, etc.) of normal sera. It is to be regretted that although in numerous instances these have been shown to consist of amboceptor and complement this fact has not been sufficiently regarded by this school (see especially the recent studies by Sachs, pages 181 et seq., and Morgenroth and Sachs, page 233).

by its behavior toward heat, completely losing its action on being heated to 56° C. for three-quarters of an hour.

Observations in this same direction have been made by Shibayama<sup>1</sup> and Klein,<sup>2</sup> and a comprehensive study by Tarashevitch<sup>3</sup> has recently appeared from Metchnikoff's laboratory.

Shibayama, working in Kitasato's laboratory, studied the action of extracts of guinea-pig organs on dog blood and obtained hæmolysis with those of spleen and lymph glands, but not with those of bone marrow and other organs. Without further analysis he classes as identical the hæmolytic substances of the organs and the specific hæmolysins which appear in the serum after immunization with dog blood-cells. This leads him to the following conclusion: "From the facts mentioned it can readily be seen that the hæmolytic side-chains of the guinea-pig are already physiologically present in the spleen and lymph-glands and that the injection of dog blood aids their hyper-production."

Klein prepared the organ extracts by crushing them with quartz gravel, then mixing with an equal amount of physiological salt solution and filtering in the cold. The only constant effect was the hæmolytic action of the extract of pancreas; in a few cases the extract of kidney and of intestinal mucosa also dissolved the red blood-cells.

Metchnikoff's experiments were continued in his laboratory by Tarashevitch, who studied principally the organs of guinea-pigs, rabbits, and dogs. Corresponding to Metchnikoff's first experiments, he tested the hæmolytic action mostly on avian blood-cells, but also on those of mammals. In the guinea-pig, in the great majority of cases, he found the extracts of omentum, mesenteric lymph-glands, and spleen to be hæmolytic. Besides this pancreas extract and in many cases salivary gland extract were hæmolytic. In general the hæmolytic action of the organ extracts of rabbits is weaker than that from the organs of guinea-pigs. Omentum, spleen, and mesenteric glands frequently were hæmolytic; the salivary glands acted feebly; bone marrow, liver, and thymus were not hæmolytic. *According to Tarashevitch, therefore, only the macrophagic organs and the digestive glands possess a hæmolytic action.*

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<sup>1</sup> Shibayama, Centralblatt f. Bact., Vol. 30, 1901, No. 21.

<sup>2</sup> Klein, K. k. Ges. der Aerzte in Wien, Sitzung von Dec. 20, 1901, reported in Wiener klin. Wochenschr. 1901, No. 52.

<sup>3</sup> Tarashevitch, Sur les Cytases, Annal. de l'Inst. Past. 1902.

If the organ extracts are heated to 56° C. for half or one hour the hæmolytic property disappears in many cases; in other cases it is diminished; very rarely it remains unchanged. According to Tarassevitsch, this variation from the "cytases" (which in general are destroyed by heating for half an hour to 56° C.) is only an apparent one. In the organ extracts the "macrocytase" is not completely liberated, but is held back to a great extent by the cell detritus present in the emulsion. It leaves the detritus only very slowly and incompletely, as is shown by the fact that the entire emulsion is always more active than the fluid portion obtained by centrifuging, and also that by filtering through paper the clear fluid is deprived of the greater part of the properties which the entire emulsion possesses. This filtered fluid, in which, according to Tarassevitsch, all the "cytases" present are in dissolved form, is said to behave toward thermal influences like hæmolytic serum.

*Finally according to Tarassevitsch the thermostability of the entire extracts is not very great. If he heated his extracts a little higher, one to two hours, to 58.5°, 60°, 62°, the hæmolytic property disappeared completely.*

From this behavior toward thermic influences Tarassevitsch concludes that the relationship of the hæmolytic substances of the organ extracts to the "cytases" of serum is perfectly clear, and that it is incorrect to ascribe a hæmolytic property which can be destroyed at such low temperatures, to osmotic phenomena or to the presence of "de quelques substances chimiques." Hence, as Metchnikoff assumed, the organs in question contain a macrocytase, and this circumstance proves that the macrophagic organs must play a rôle in the formation of the natural and the artificial hæmolysins.

In the following pages we shall describe certain experiments in which we have reached essentially different results from those obtained by Metchnikoff and Tarassevitsch.

The emulsion of the organs was prepared as follows: The organs removed from the exsanguinated animals are rubbed up very finely with sea-sand which has first been purified with hydrochloric acid. Then 5 to 10 times their weight of physiological salt solution is added and the mixture thoroughly shaken in a shaking-machine for two hours, whereupon the coarser particles are removed through several hours' centrifuging. A more or less uniformly clouded fluid remains. The organ extracts were employed as fresh as possible, though it was found that they could well be preserved by freezing them at -10° to -15° C.<sup>1</sup>

<sup>1</sup> On thawing them out we often observed the appearance of numerous

In studying the hæmolytic action blood-cells were used which had been freed from serum as much as possible.

The series of tubes was kept in the thermostat at 37° C. for two to three hours and overnight in the refrigerator at 8° C. In the presence of large amounts of organ extracts hæmolysis proceeds rapidly; with small amounts it is very slow. The tubes must be frequently shaken while being kept at 37°; the result can only be judged of on the following day.

To begin we sought to gain a general idea of the hæmolytic action of several organ extracts on various species of blood. The extracts of intestine and of stomach of the mouse as well as that of the stomach of guinea-pigs and of the pancreas of oxen always showed a strong hæmolytic action on all species of blood which we examined, 1.0 cc. to 0.5 cc. of the extracts sufficing to completely dissolve 1 cc. 5% blood of rabbit, guinea-pig, mouse, rat, goat, sheep, ox, pig, horse, dog, or goose. The rest of the organ extracts examined, namely guinea-pig intestine, rat intestine, rat stomach, varied in their hæmolytic property with different bloods, qualitatively as well as quantitatively. Extract of guinea-pig spleen dissolved only dog blood and guinea-pig blood; extract of mouse spleen possessed a feeble hæmolytic action on guinea-pig blood and pig blood. Extract of guinea-pig adrenals dissolved both the blood species examined in this case, viz., guinea-pig blood and goose blood. We found the extract of spleen, mesenteric lymph nodes, pancreas, stomach, intestine, and adrenals of one dog to be strongly hæmolytic for guinea-pig blood, whereas in another case the spleen showed itself absolutely inert, although the pancreas was strongly hæmolytic. This variation in the hæmolytic action on various blood-cells has already been noticed by other investigators, and we therefore desire merely to call attention to a point which thus far has not been regarded, namely, *that the organ extracts are able to dissolve the blood-cells of the same species and even of the same individual from which they are derived.*

Thus according to our experience emulsions of guinea-pig stomach, spleen, adrenal, kidney, and intestine, of mouse intestine and stomach, of rat intestine and stomach, of ox pancreas, dissolve the red blood-cells of their own species. The relation existing between this action on the blood of the same species and hæmolysis of foreign species of blood is shown by the following two experiments. (See Table I.)

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clumps in the organ extracts which before had been free from visible particles. These clumps could be separated by centrifuge, and exhibited a hæmolytic action when suspended in salt solution.



TABLE I.  
EMULSION OF MOUSE INTESTINE (10%).

	1 cc. 5% Ox Blood.	1 cc. 5% Guinea- pig Blood.	1 cc. 5% Mouse Blood.
1.0	complete	complete	complete
0.75	"	"	"
0.5	almost complete	"	"
0.35	trace	"	"
0.25	0	"	trace
0.2	0	"	0
0.15	0	"	0
EMULSION OF BEEF PANCREAS (10%).			
	1 cc. 5% Rabbit Blood.	1 cc. 5% Guinea- pig Blood.	1 cc. 5% Ox Blood.
0.5	complete	complete	complete
0.35	"	0	0
0.25	strong	0	0
0.15	0(?)	0	0

These experiments show that the susceptibility of the body's own blood may be very great, even as great as that of a foreign species of blood. Whether *all* these extracts dissolve the blood of the own individual we have not determined; we regard it as probable, however, since positive results were obtained in all experiments which we made in this direction, especially with extracts of mouse intestine and of guinea-pig stomach.

These experiments (especially those with the extract of guinea-pig spleen, which Shibayama too found to be active only for dog blood) show that we are *not here dealing with hæmolytic poisons of a general kind* (such as saponin, the gallic acid salts, and certain alkaloids, like solanin, which dissolve all blood-cells regardless of species), but that these hæmolytic poisons *possess a certain specificity* which is of special biologic interest.

The property of organ extracts to dissolve the blood-cells from the same individual is of great significance because neither when normal nor after immunizing procedures does the *blood-serum* of these animals ever contain substances which damage the blood-cells of the animal itself (autohæmolysins). Tarassevitch himself noticed the great difference existing, on the one hand, between the absence of a marked hæmolytic action of *guinea-pig serum* on foreign species of blood and the strong hæmolytic action of the *extracts of certain guinea-pig*

*organs*, on the other. He believes to explain this by assuming a difference in the macrocytase extracted from the organs and that present in the serum. In any case this constitutes a serious dilemma for Tarashevitch; for either there are several "macrocytases" as opposed to the unitarian view of Metchnikoff or the macrocytase of serum is identical with that of the organ extracts. In view of this entirely different behavior, however, the latter does not appear acceptable to Tarashevitch.

Our first question was an entirely different one, for in all the cases of hæmolysis and bacteriolysis sufficiently examined we had never met with a simple alexin in the sense of Buchner and Metchnikoff, but invariably found a coaction of amboceptor and complement. In view of this our investigations had, above all, to determine whether the hæmolytic organ extracts could be shown to be characterized by complement and amboceptor.

These first doubts, namely, whether these substances corresponded to what we conceive as the complex hæmolysins of blood-serum, led us to study the hæmolytic organs in respect to those main characteristics which we have come to know in our study of the complex hæmolysins. These are: 1. The behavior toward thermic influences. 2. The behavior when bound to the red blood-cells at low temperatures. 3. The power of producing antibodies by immunization.

We shall begin by describing a number of typical experiments which show the behavior of the organ extracts toward higher temperature. Let us glance first at the experiments dealing with the effect of organ extracts on goose blood-cells, for this is the blood species which has been mainly used by Metchnikoff and Tarashevitch. (See Table II.)

These experiments clearly show that in most of the cases the hæmolytic action of organ extracts on goose blood-cells is not at all or but slightly affected by a three-hour heating, to 62° C., and that heating to 100° C. for one hour and even for three hours does not produce any further damage. Only the hæmolytic effect of extract of mouse intestine is reduced to about one-half by the heating to 62° C.; heating to 100° C. for three hours causes but little additional damage. But that this cannot be a true destruction of part of the hæmolysin will be discussed later. We wish next to present additional experiments dealing with the behavior of heated organ emulsions on guinea-pig blood. (See Table III.)

Nor is this result changed if stronger agents, such as alkalies or acids, are employed at high temperatures. (See Table IV.)

TABLE II.

## A. ACTION OF HEATED ORGAN EXTRACTS ON GOOSE BLOOD-CELLS (1 cc. 5%).

I. *Extract of Dog Spleen* (10%).

	Not Heated.	3 Hrs. (62°).
0.2	complete solution	complete solution
0.15	“	almost complete
0.1	very little	very little to trace

II. *Extract of Dog Stomach* (10%).

	Not Heated.	3 Hrs. (62°).	1 Hr. (100°).	3 Hrs. (100°).
0.35	complete	complete	complete	complete
0.25	“	“	“	“
0.15	“	“	“	“
0.1	very little	very little	very little	very little

III. *Extract of Dog Pancreas* (10%).

	Not Heated.	3 Hrs. (62°).	1 Hr. (100°).	3 Hrs. (100°).
0.75	complete	complete	complete	complete
0.5	“	“	“	fairly complete
0.35	strong	0	0	0
0.25	very little	0	0	0
0.15	0	0	0	0

IV. *Extract of Dog Mesenteric Lymph Glands* (10%).

	Not Heated.	3 Hrs. (62°).	1 Hr. (100°). <sup>1</sup>	3 Hrs. (100°). <sup>1</sup>
0.75	complete	complete	complete	complete
0.5	“	“	strong	strong
0.35	“	almost complete	very little	very little

<sup>1</sup> Enormous coagula.V. *Extract of Mouse Intestine* (5%).

	Not Heated.	3 Hrs. (62°).	1 Hr. (100°).	3 Hrs. (100°).
0.35	complete	complete	complete	complete
0.25	“	“	“	moderate
0.2	“	strong	strong	“
0.15	“	moderate	little	little
0.1	almost complete	little	trace	trace

TABLE III.

ACTION OF HEATED ORGAN EXTRACTS ON GUINEA-PIG BLOOD (1 cc. 5%).

I. *Extract of Dog Mesenteric Glands (5%).*

	Not Heated.	1 Hr. (64°).	30 Hrs. (100°).
0.25	complete	complete	complete
0.15	"	"	"
0.1	trace	0	faint trace
0.075	0	0	0

II. *Extract of Ox Pancreas (10%).*

	Not Heated.	1 Hr. (62°).
0.35	complete	complete
0.25	"	"
0.15	strong	strong

III. *Extract of Ox Pancreas (20%).*

	Not Heated.	1 Hr. (68°).	1½ Hrs. (100°).
0.15	complete	complete	complete
0.1	"	"	"
0.075	trace	trace	trace
0.05	0	faint trace	0

IV. *Extract of Guinea-pig Stomach (10%).*

	Not Heated.	3 Hrs. (65°).
0.25	complete	complete
0.2	"	"
0.15	strong	strong

TABLE IV.

EXTRACT OF OX PANCREAS (10%).

	Not Treated.	Containing 1/50 n. HCl Heated to 60° for 30 Min. and Neutralized	Containing 1/50 n. NaOH Heated to 60° for 30 Min. and Neutralized.
0.35	complete	complete	complete
0.25	"	almost complete	almost complete
0.15	faint trace	0	0
0.1	0	0	0

All these experiments show that the organ extracts will bear heating to 62–68° C. for hours, and even 100° for several hours, without suffering any change in their hæmolytic properties worth mentioning. In these experiments, in fact, we have been unable thus far to find any limit for the thermostability of the organ extracts. We are therefore dealing with substances which withstand boiling (coctostabile), and this fact in itself is sufficient to disprove the assumption that they are “cytases.”

The next question, of course, is how such a fundamental divergence between our results and those from Metchnikoff's highly esteemed laboratory can be explained. We think we have discovered the cause of this difference. It is as follows:

In the above experiments it is of the greatest importance to shake the fluid previous to testing its hæmolytic property; in that way the more or less plentiful precipitate formed on heating is again uniformly distributed throughout the fluid. *Only the coagulum* produced by heating possesses a hæmolytic action. According to our experience, if a precipitate has been produced through heating, the *clear fluid* which is separated from this no longer possesses *any hæmolysin whatever*. If the precipitate is separated by centrifuge the clear fluid will be found inert; on suspending the sediment in the requisite quantity of physiological salt solution a new emulsion is obtained which has preserved the hæmolytic property. This is shown in the following table.<sup>1</sup>

According to these experiments it would seem very probable that the contradictory results obtained by us on the one hand and by Metchnikoff and Tarassevitsch on the other are due to insufficient regard being paid by the latter to the precipitates formed in the organ extracts on heating.

If we assume that the hæmolytic, coctostable substance is present

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<sup>1</sup> The coagula formed on heating may be so plentiful that they render an exact observation of hæmolysis exceedingly difficult. It is frequently seen that hæmolysis by means of heated organ extracts which are filled with coagula proceeds very slowly; apparently the precipitates offer considerable resistance to the escape of the hæmolytic substance. Naturally, this constitutes a source of error, since with low temperature and too short a time for observation the hæmolytic action is underrated. This may also explain the occasional weakening of heated organ extracts, to which we have already referred; in that case the weakening would not be due to a partial destruction of the hæmolytic substance.

TABLE V.

## I. EXTRACT OF DOG LYMPH GLANDS (10%).

Guinea-pig blood (1 cc. 5%).

	Fresh.	1 Hr. (62°). (No Coagulum.)	1 Hr. (100°). Slight Precipitate, Centrifuged, and Suspended in Salt Solution.	1 Hr. (100°). The Clear Fluid obtained by Centrifuging.
2.0	—	—	complete	0
1.5	—	—	"	0
1.0	complete	complete	"	0
0.75	"	"	—	0
0.5	"	"	complete	—
0.25	"	strong	—	—
0.15	strong	very little	—	—

## II. EXTRACT OF DOG PANCREAS (20%).

Guinea-pig blood (1 cc. 5%).

	Fresh.	1 Hour (62°). (No Coagulum.)	1 Hr. (100°). Slight Precipitate. Centrifuged, and Suspended in Salt Solution.	1 Hr. (100°). The Clear Fluid obtained by Centrifuging.
2.0	—	—	complete	0
1.5	—	—	"	0
1.0	complete	complete	"	0
0.75	"	"	—	0
0.5	"	little	moderate	0
0.25	little	0	—	—
0.15	"	0	—	—

## III. EXTRACT OF DOG INTESTINE (10%).

Goose blood (1 cc. 5%).

	1 Hr. (100°). Precipitate again Uniformly Distributed.	1 Hr. (100°). Precipitate after Centri- fuging, Suspended in Salt Solution.	1 Hr. (100°). Centrifuged Fluid still somewhat Cloudy.
1.5	complete	complete	little
1.0	"	"	trace
0.75	"	"	0
0.5	"	"	0
0.35	almost complete	almost complete	0
0.25	—	0	0
0.2	—	—	0
0.15	• —	—	0

TABLE V—*Continued.*

## IV. EXTRACT OF MOUSE INTESTINE (10%).

Goose blood (1 cc. 5%).

	3 Hrs. (100°). Precipitate again Uniformly Distributed.	3 Hrs. (100°). Precipitate Suspended in Salt Solution.	3 Hrs. (100°). Clear Centrifuged Fluid.
1.0	complete	—	0
0.75	“	complete	—
0.5	“	“	0
0.35	“	strong	0
0.25	moderate	trace	—
0.2	“	“	—
0.15	little	faint trace	—
0.1	very little	minimal	—

in the organ extracts in dissolved form we find it difficult to understand the fact that it is abstracted from the fluid by means of the coagulum formed on heating. To be sure, one could think of an absorption by the coagulum. The complete abstraction by means of heating is, however, readily understood if the hæmolytic substance is present, not in solution, but in a state of finest suspension; for it is a matter of common experience that substances finely suspended in a fluid are carried down with a precipitate produced in the fluid. The technique of clearing cloudy fluids rests to a large extent on such precipitations.

We have not yet been able to decide definitely whether the hæmolytic substance is present in the fluid in dissolved form or in very fine suspension; we incline strongly to the latter view. We base this (1) on numerous experiences which show that by filtering the organ extracts through porous filtering candles the fluid obtained is entirely inert; (2) on the behavior of the hæmolytic substance when treated with alcohol.

One part of a 1% extract of ox pancreas is mixed with ten parts 96% alcohol, and after a time the fluid is filtered off from the flaky precipitate which has formed. The entirely clear filtrate is distilled in vacuo and the portion left behind mixed with physiological salt solution. A coarsely flocculent suspension is thus obtained which possesses strong hæmolytic action, about one-half to one-third of the original strength. If this mixture is now filtered, the clear filtrate is found to be absolutely inert, whereas the flakes washed from the filter exhibit almost the full hæmolytic effect. The following experiment will serve as an example.

TABLE VI.

GUINEA-PIG BLOOD (1 cc. 5%), EXTRACT OF OX PANCREAS (10%). PORTION LEFT FROM THE ALCOHOLIC DISTILLATE SUSPENDED IN 0.85% SALT SOLUTION.

	Total Fluid.	Clear Filtrate.	Suspension of the Flakes.
1.0	complete	0	complete
0.5	"	0	"
0.35	—	0	"
0.25	complete	0	"
0.15	—	0	strong
0.1	moderate	0	trace

We are therefore evidently dealing with a substance which in the above treatment is dissolved in the alcoholic fluid but which is soluble to only a very slight degree in salt solution.

Naturally a certain degree of solubility is always one of the conditions of the hæmolytic action observed, but this need only be a minimal one. The blood-cells can anchor the amount of hæmolytic substance in solution at any given time and so render the fluid capable of taking up small amounts of the substance anew. This conception of a relative insolubility of the substance is readily reconciled with the hæmolytic action. The process which takes place reminds one of that occurring with certain dyes, which, although not given off to the water from the dyed fibre, are nevertheless able by means of the watery medium to go from the dyed to undyed fibres.

The coctostability of the hæmolytic substances of organ extracts, their adherence to solid particles, their solubility in alcohol—all these, in our opinion, show that these substances cannot be classed as identical either with the "cytases" of Metchnikoff or with our complex hæmolysins. Nevertheless we have still further examined these substances for properties which characterize the hæmolysins.

In one case, therefore, we studied the action of our organ emulsion on blood-cells at 0° C. in order to determine the possibility of separating a possible amboceptor and complement.

To each 1 cc. of a 5% suspension of guinea-pig blood which had been thoroughly cooled on ice, varying amounts of cooled extract of ox pancreas were added and the mixture kept at 0° for two hours and frequently shaken. In this case slight solution occurred only with large quantities of the extract. Then the mixtures were centrifuged, the sediment resuspended in salt solution (1.5 cc.), and the



decanted fluid mixed with 0.05 cc. of guinea-pig blood freed from serum. (See Table VII.)

TABLE VII.  
GUINEA-PIG BLOOD (1 CC. 5%).

Pancreas Extract.	Solution at the End of Two Hours at 0°.	Hæmolysis with the Decanted Fluid.	Hæmolysis of Sediment.	Control, Absolute Action in Warmth.
1.0	little	complete	complete	complete
0.5	0	0	"	"
0.35	0	0	"	"
0.25	0	0	almost complete	"
0.15	0	0	strong	strong

We see, therefore, that at 0° the single solvent dose has been completely anchored by the blood-cells and that after centrifuging this leads to complete solution at higher temperatures; double the solvent dose is still completely anchored by the blood-cells. This condition of affairs does not at all correspond to the behavior of the complex hæmolysis of serum.

It still remained to study another fundamental characteristic, namely, the *formation of antibodies*.

We made peritoneal injections into rabbits, using for this purpose a strongly active extract of ox pancreas that had been sterilized by heating to 60° C. for one hour. The precipitate which developed being regarded as the true active constituent, the mixtures were thoroughly shaken and the whole injected. Two rabbits received 20 cc., 45 cc., and 60 cc. of the extract at suitable intervals and were bled ten days after the last injection. The anti-hæmolytic action of the serum against the extract was found to be exactly the same as that of normal rabbits. (See Table VIII.)

As can be seen from this experiment (the result of which is confirmed by a number of similar experiments with the serum of other rabbits and of a goat treated in like manner) it has not been possible to produce antibodies by injections of pancreas extract.

The experiment, moreover, shows that normal rabbit serum already possesses a marked inhibiting action on the hæmolysis through organ extracts.<sup>1</sup> We have been able to demonstrate this on all the species

<sup>1</sup> This action of the serum must always be taken into account in the experiments, and the blood-cells first washed.

TABLE VIII.

1 cc. GUINEA-PIG BLOOD + 0.5 EXTRACT OF OX PANCREAS = TWICE  
THE SOLVENT DOSE.

	+ Serum. cc.	1. Of Rabbits Immunized with Pancreas Extract Inactive.	2. Of Normal Rabbits Inactive.
1	0.25	0	0
2	0.2	0	0
3	0.15	0	0
4	0.1	complete	almost complete
5	0.075	"	complete
6	0.05	"	"
7	0.015	"	"

of sera investigated by us; it is especially marked in ox serum, as can be seen by the following examples. (See Table IX.)

TABLE IX.

1 cc. 5% Guinea-pig Blood + 0.5 cc. Extract of Ox Pancreas.

cc.	+ Inactive Rabbit Serum.	+ Inactive Goat Serum.
1.0	0	0
0.5	0	0
0.25	0	0
0.1	almost complete	0
0.05	complete	strong
0.025	"	complete
0	"	"
Guinea-pig Blood, 1 cc. 5% + Extract of Ox Pancreas, 1 cc. (= 4 times the solvent dose) + Inactive Ox Serum ( $\frac{1}{2}$ hour at 56° C.).		
0.05		0
0.025		0
0.01		strong
0		complete

That these antihæmolytic actions of normal sera are not due to antibodies in the proper sense is shown by the fact that this protective action withstands the action of high temperature, even 100° C. This is shown by the following table.

TABLE X.

	Extract of Pancreas.  cc.	1 cc. 5% Guinea-pig Blood + 0.2 cc. (1 cc. = $\frac{1}{5}$ ) Goat Serum.		
		Goat Serum was Heated for 1 Hour		Without Serum.
		at 70°.	at 100°.	
1	1.0	complete	complete	complete
2	0.75	trace	faint trace	"
3	0.5	0	0	"
4	0.35	0	—	"
5	0.25	0	—	"
6	0.15	0	—	faint trace
7	0.1	0	—	0
8	0	0	—	0

The serum was diluted five times with tap-water and after heating the corresponding amount of salt was added.

This experiment shows that the goat serum, which in amounts of 0.2 cc. almost completely neutralizes three times the solvent dose of the emulsion, does not suffer the slightest loss of action even when heated to 100° C. for one hour; that an antibody in the proper sense is, therefore, not present.

Whether the *coctostable* substance which acts here is a simple unit which acts specifically on the hæmolytic substance of the organ extract, or whether we are dealing with a complex of bodies having an "antireactive" action, can only be determined by further investigations.<sup>1</sup>

The hæmolytic substances found in organ extracts and examined by us are, therefore,

1. *Coctostable*;
2. *Soluble in alcohol*;
3. *Not complex*;
4. *Not able to excite the production of antibodies*.

This shows that we are dealing with substances which are entirely distinct from the hæmolysins of serum and which belong into a peculiar class of substances acting hæmolytically.

<sup>1</sup> Analogous actions of coctostable substances have recently been observed by Korschun, who has described a "pseudo-antirennin" of normal sera (Zeitschr. f. physiol. Chemie, Vol. 36, Nos. 2 and 3, 1902). A thermostable substance inhibiting the action of urease has also been recently described by Moll (Hofmeister's Beiträge, Vol. II, Nos. 7-9).

These substances show a certain analogy to the bactericidal bodies obtained by Conradi<sup>1</sup> in the autolysis of organs, since the latter are also coctostable and soluble in alcohol. In contrast to the former, however, Conradi's substances pass through porous filters.

At present it is impossible to say whether these substances are already preformed in the living cell or whether they originate only on the disintegration of the living protoplasm either through destruction of the cells or through the influence of the extracting agents. The presence of amboceptors and complements in the living cell is in no way prejudiced by this demonstration. In the future, however, the sources of error pointed out by us must be taken into account in drawing conclusions.

One thing must be regarded as certain, that these experiments disprove the identity of the hæmolytic substances in question and the "cytases" in the complements of serum.

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<sup>1</sup>Conradi, Beiträge zur chem. Physiol. in Pathol., Vol. 1, Nos. 5 and 6, 1901.

## XXVI. REVIEW OF BESREDKA'S STUDY, "LES ANTI-HEMOLYSINES NATURELLES."<sup>1</sup>

By H. T. MARSHALL, M.D., and Dr. J. MORGENROTH.<sup>2</sup>

THE chief result of Besredka's study is the following conclusion: The serum of sick and healthy persons contains an *antihæmolysin*, in the form of a *simple antiamboceptor*, which acts *exclusively* on the specific amboceptor fitting human blood. The amboceptor used in this author's experiments, and conceived as strictly unitarian, was derived from a goat treated with human blood. Antihæmolysins which protect the blood-cells of species *other* than man against hæmolysins are not present in human serum, and the rule that the normal antihæmolysin, i.e., the antiamboceptor of a serum, always protects only its own blood-cells, is of general application.

It was easy for us to show by experiments that the last generalization is entirely untenable. The most varied kinds of sera (thus especially horse serum) protect human blood-cells against specific hæmolysins,<sup>3</sup> and conversely, according to our experiments, human serum protects ox blood-cells.

It is absolutely necessary, above all, to get the two false premises out of the way which give rise to all of Besredka's mistakes. This is a simple matter, for these premises were possible only because the experiments which had long since shown them to be untenable were ignored. The two erroneous premises are:

1. All the amboceptors obtained by injecting any species whatsoever with a particular species of blood are entirely identical. Thus Besredka assumes that if different species, e.g., rabbits, guinea-pigs,

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<sup>1</sup> Annal. de l'Institut Pasteur, Oct. 1901.

<sup>2</sup> From a detailed study, "Über Anticomplemente und Antiamboceptoren normaler Sera und pathologischer Exsudate," appearing in Zeitschrift für klinische Medizin, where the experimental part is to be found.

<sup>3</sup> See our experiments in Zeitschr. für klin. Medizin, Table III.

goats, are injected with blood-cells of a different species, say man, the amboceptors developed will all be identical.

2. Hæmolysis of foreign species of blood by normal sera is due exclusively to the presence of a *single, simple alexin*, and not to a complex hæmolysin consisting of amboceptor and complement.

The thorough studies of Ehrlich and Morgenroth positively prove the incorrectness of the first assumption. Above all, these investigations showed that that body in a hæmolytic immune serum, which we term *the amboceptor*, can, in one and the same animal, be shown experimentally to be made up of a *host of different kinds of amboceptors*. Furthermore, by means of combining experiments, of experiments with an artificially produced antiamboceptor, and by studies on the complementibility of amboceptors of different animal species it was shown that amboceptors directed against the same species of blood, which are obtained from different animal species, differ not only in their complementophile group but also in their cytophile group.

Besredka, who only learned of this study after the completion of his own, regrets that "étant donné la complexité de plus en plus grande de la question, de ne pas pouvoir suivre ici les auteurs dans leur argumentations." It would be deplorable if the principle should gain ground that the results of other workers can simply be ignored on the plea that the verification of the experimental evidence is rather difficult owing to its complexity. Finally, the diversity of the amboceptors has already been established by the studies on isolysins.<sup>1</sup> In this it was shown that even with twelve goats treated with goat blood, twelve different isolysins are to be distinguished, i.e., twelve amboceptor complexes against the same species of blood.

This large number of amboceptors fitting one blood-cell corresponds to a like condition of the blood-cell's receptors. These must be extraordinarily manifold, because, besides the receptors which anchor the amboceptors, there are present the most varied receptors for the numerous simple hæmolysins and hæmagglutinins. This view, enunciated in detail by Ehrlich,<sup>2</sup> has recently been confirmed by the experiments of Landsteiner and Sturli.<sup>3</sup> These authors showed that blood-cells which have been completely saturated with the

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<sup>1</sup> Ehrlich and Morgenroth. (See page 88.)

<sup>2</sup> Ehrlich, Nothnagels Spec. Pathol. u. Therapie, Vol. VIII, 1901.

<sup>3</sup> Landsteiner and Sturli, Über die Hæmagglutinine normaler Sera. Wiener klin. Wochensh. 1902, No. 2.

agglutinin of one normal serum can still take up in succession the agglutinin of a second, third, and even fifth serum in any order one chooses. Thus the agglutinin of horse serum was still bound by pigeon blood-cells which had been treated with goat and rabbit serum to such an extent that the cells were unable to abstract any more agglutinin from these sera. These results are only comprehensible if one assumes a large number of different receptors for the agglutinins of different sera, and it is therefore surprising to find that just these experiments which harmonize so well with Ehrlich's views should be given a different and complicated interpretation by Landsteiner and Sturli.

Besredka's second premise likewise does not correspond to the facts. It is now three years since Ehrlich and Morgenroth (see page 11) demonstrated the complex nature of normal hæmolysins in a number of cases; later they brought forward evidence in favor of the plurality of complements. In a final study on this subject Sachs has recently (see page 181) shown that in those cases in which other investigators did not succeed in demonstrating the complexity of normal hæmolysins only technical difficulties and experimental errors were to blame.

After this brief analysis of the principles involved, we can proceed to study Besredka's experiments and discuss his conclusions from the same.

The case especially investigated by Besredka deals with the combination human blood + amboceptor of a goat immunized with human blood and guinea-pig serum as complement. If inactive human serum is added to this combination, solution will be prevented, as we were able to verify. From this behavior of the human serum Besredka concluded that this must contain an *antiamboceptor*, giving the following as his reasons.

According to Besredka the serum of each particular animal species contains a single, simple "cytase" specific for this animal. This author has now sought to determine whether human serum as such contains an "anticytase" against the "cytase" in question; in other words, whether in this case the inactive human serum contains an anticytase against guinea-pig serum. The solution of this problem was extremely easy for Besredka. Guinea-pig serum, as we know, dissolves certain species of blood, and does so only by means of its "cytase." This action is not inhibited by human serum. Hence

human serum contains no anticytase whatever, and when, as in the above combination, human blood+specific amboceptor+guinea-pig serum, this serum exerts a protective action, it follows by exclusion that this action is due to an antiamboceptor.

The fundamental error in this method of proof lies, as already mentioned, in the assumption of a simple cytase, which cytase, moreover, is able by itself to effect hæmolysis. As a matter of fact, however, solution of the blood-cells by guinea-pig serum is brought about only by this, that the blood-cells combine with a normal amboceptor present in the blood serum, and that this thereupon anchors the complement (cytase) which effects solution. If the complement in itself is conceived as a single substance, one could conclude from the fact that the human serum does not prevent\* this normal hæmolysis that the human serum contains neither an antibody against the normal amboceptor nor against "the complement." In reality, however, "the complement" is made up of numerous partial complements, one or another of which is dominant for the completion of particular amboceptors, be these hæmolytic or bacteriolytic. This theory of dominant complements has been firmly established by Ehrlich and Marshall.<sup>1</sup>

It has already been proven for anticomplementary sera that such a serum neutralizes only part of the complements of a second serum, not all. Marshall and Morgenroth<sup>2</sup> have shown that the anticomplement of a human ascitic fluid prevents the complementing action of guinea-pig serum for one hæmolytic amboceptor leaving that of another intact.

Now Besredka showed that human serum does not prevent the normal hæmolytic action of a certain serum, although it acts anti-hæmolytically when this is used as complement for an amboceptor produced by immunization. The only conclusion to be drawn from this is that the human serum contains no anticomplement which acts against the complement dominant in the case of the normal hæmolysis. This, of course, does not prevent the same serum from acting on other partial complements which are dominant in other cases. We see, therefore, that Besredka's entire method of proof lacks a firm basis.

It is further to be remembered that such questions are to be de-

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<sup>1</sup> See page 226 et seq.

<sup>2</sup> See page 222.



cided not by pure speculation but by experimental means. The centrifugal method allows us to demonstrate antiamboceptor and anticomplement directly, as such, entirely independent of all theoretical speculations. In the case here described, we have shown that an anticomplement action is present almost exclusively, compared with which the slight antiamboceptor action is of no account.<sup>1</sup>

As a result of our own results we must maintain, first, that the major part of the anti action of the human serum described by Besredka is due to the anticomplement; second, that Besredka's experimental method allows no conclusions whatever regarding an anti-immune body; and third, that the part played by the individual factors in this antihæmolytic action can only be decided by the method employed by us.

After having, then, as a result of the experiments with human blood, erroneously ascribed the antihæmolytic action to an antiamboceptor, Besredka continues his study by investigating whether this supposed antiamboceptor is specific, i.e., only for human blood and serum dissolving human blood. In this sense he arrives at a positive conclusion. His generalization is based on the following observation: He finds that human serum does not protect sheep blood against the hæmolytic serum of a goat immunized with sheep blood, the hæmolytic serum being reactivated with guinea-pig serum. We have made the same observation and studied just this behavior by means of a human ascitic fluid. The case in question, however, constitutes a special exception, due to a partial anticomplement, and it is, therefore, peculiarly unsuited as the basis for a generalization. Our experiments show that on investigating other cases, human serum is found to exert a considerable protection against normal hæmolysins and those produced by immunization which dissolve other species of blood—ox blood in our case. Here also, however, this protection is due to anticomplements and not to antiamboceptors, at least so far as can be determined by an exact analysis.

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<sup>1</sup> The destruction and weakening of the antihæmolysin which Besredka shows occurs with longer heating to 65–67° C. is, of course, in no way characteristic for the nature and mode of action of the antibody. We showed that this temperature injures both antiamboceptor and anticomplement. Besides, the behavior toward narrowly limited thermal influences does not possess the significance of a group reaction. This is well shown by the occurrence of a thermostable complement (Ehrlich and Morgenroth, page 11) and thermolabile amboceptors (Sachs, see page 181).

Finally, the fact that at times a small part of the antihæmolytic action (as in our experiments with a human exudate and ox blood) is due to an antiamboceptor, removes the basis for Besredka's generalization that a normally present antiamboceptor always protects only its own blood-cells.

From all this it follows that the part believed to be played by the antiamboceptors of human and animal body fluids in the prevention of hæmolysis is materially decreasing in favor of the part taken by the anticomplement. There is no doubt at all that antiamboceptors exist in normal serum; this was first proven some time ago by Ehrlich and Morgenroth,<sup>1</sup> and also by P. Müller.<sup>2</sup> These antiamboceptors do not, however, occur regularly, as was also pointed out at that time.

Our analysis therefore shows that since the fundamental fact does not apply, the extensive theoretical conclusions drawn by Besredka from the exclusive protection of the homologous blood-cells in the serum cannot be recognized. That the amboceptors present actually primarily protect the blood-cells of the corresponding species is probable in itself, for according to our view, as mentioned elsewhere, they represent free cell receptors. Besredka assumes that the reason for the development of his supposed antiamboceptors is this: the blood-cells, which are constantly dying in the organism, cause the production of hæmolysins. These would endanger life if the organism did not paralyze their action through the development of antiamboceptors. Such a regulating contrivance can surely not be very common, since it was not observed by Ehrlich and Morgenroth in the numerous experiments on isolysins, in which it would most readily have been discovered. But if such a contrivance were a necessity, it would have to be *constant*. This, however, is not at all the case as we have already established.<sup>4</sup>

The simplest and most natural assumption is that the antiamboceptors are nothing else than products of cell disintegration, free receptors which are capable of binding amboceptors and so exert a deflecting influence. The assumption that these free receptors are products of retrogressive metabolism is borne out by the fact that

<sup>1</sup> See page 88 et seq.

<sup>2</sup> P. Müller, *Centralblatt f. Bakt.*, Vol. 29, 1901.

<sup>3</sup> Morgenroth. (See page 241 et seq.)

<sup>4</sup> See pages 23 and 71 et seq.

lished by Schattenfroh<sup>1</sup> that they are excreted through the urine in considerable amounts.

One reason above all has led us to believe that Besredka's views required to be controverted in detail, namely, the fact that they maintain the unitarian conception that only one hæmolysin is possible against a given species of blood and one bacteriolysin against a given species of bacterium. This conception can seriously retard the development of the doctrine of immunity and especially of the practical application of this doctrine.

The recent investigations which have demonstrated the exceeding multiplicity of the cell's receptors and of the amboceptors obtained by immunizing with these receptors show that this study can be successfully pursued along two directions. One of these consists in the production of polyvalent sera by immunizing with numerous strains of the same bacterial species. It may be assumed that the varieties of a bacterial species contain the various receptors in very varying proportions, and this is confirmed especially by Durham's experiments concerning the agglutinability of different strains of coli by specific sera. A sufficient increase of all the amboceptor types in question is therefore possible only after a high degree of immunization has been effected against a large number of related strains. This procedure had previously been chosen by Denys and van de Velde in the production of their polyvalent streptococcus serum, and has recently been employed by Wassermann and Ostertag<sup>2</sup> for the preparation of an effective serum against hog cholera. These procedures are based entirely on the experiments of Ehrlich and Morgenroth, just mentioned.

The other method of obtaining effective bactericidal sera is based on the assumption that the amboceptors, according as they are derived from different animal species, differ from one another. So far as this point is concerned, we may refer to the statements of Ehrlich and Morgenroth,<sup>3</sup> which are summed up in the sentence, "it would therefore be advisable not to attempt the production of bactericidal sera from a single animal species as is now customary, but to make

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<sup>1</sup> Schattenfroh, Münch. med. Wochensch. 1901, No. 31.

<sup>2</sup> Wassermann and Ostertag, Monatsch. f. prakt. Thierheilk, Vol. 13, foot-notes.

<sup>3</sup> See page 110.

a preparation containing a mixture of immune sera derived from animals whose receptor apparatus are as divergent as possible."

Practical results along these lines have already been achieved by Schreiber,<sup>1</sup> who made a hog-cholera serum from horses and oxen; and recently Römer,<sup>2</sup> by paying attention to just this point, has obtained an effective pneumococcus serum by utilizing several different species of animals. In view of these attempts to apply the above principles practically, it would be regrettable if the untenable unitarian view maintained by Besredka were to hinder the further development of these methods.

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<sup>1</sup> Schreiber, Berlin thierarztl. Wochenschr. 1902, No. 19.

<sup>2</sup> Römer, v. Graefe's Archiv f. Ophthalmologie, Vol. 54, 1902.

## XXVII. THE MODE OF ACTION OF COBRA VENOM<sup>1</sup>

By FUMIO KARA, A.M., M.D., Associate in Anatomy, University of Chicago,  
Fellow of the Rockefeller Institute for Medical Research.

### I. Concerning the Amboreceptors of Cobra Poison.

THE most important contributions in recent years to our knowledge of the action of animal poisons are the recently published investigations of Flexner and Noguchi<sup>2</sup> on hemolysis by means of snake venom. These authors have found that although red blood-cells whose serum has been completely removed by washing with salt solution are agglutinated by snake venom, they are not dissolved. If, however, serum is added to the washed blood cells, or if unwashed blood is used, hemolysis ensues. From this Flexner and Noguchi conclude that the hemolytic action of the snake venom is due to two factors. One of the components is contained in the snake venom itself, and is said to bear heating to about 90° C. very well. The other component is a constituent of the serum; to a certain extent this activates the poison which in itself has no action.

Flexner and Noguchi therefore arrive at the conclusion that snake venom is made up of a number of substances, acting after the manner of amboreceptors, which are activated by certain complements of the serum.

The great significance of this interesting fact is at once evident. While formerly snake venom was regarded as a simple poison acting after the manner of toxins, this shows that the hemolytic action of snake venom is somewhat more complex, being identical with the mechanism of the hemolysins of blood serum, as this has been conceived by Ehrlich and Morgenroth. For this reason Flexner and Noguchi's discovery was hailed with especial delight here in the Frankfurt Institute.

<sup>1</sup> Reprint from the Berl. klin. Wochenschr. 1902, Nos. 38 and 39.

<sup>2</sup> S. Flexner and H. Noguchi, Snake Venom in relation to Hemolysis, Bacteriolysis, and Toxicity. Journ. of Exper. Medicine, Vol. VI, No. 3, 1902.

In view of the exceeding importance of these questions it seemed advisable to proceed from these new facts and attempt to penetrate more deeply into the mechanism of the snake venom's action. We had at our disposal two specimens of dried cobra poison the hemolytic strength of which had proved to be almost identical and for which we are indebted to Dr. Lamb and Prof. Calmette.

A one per cent solution of the dried cobra poison in 0.85% salt solution served as our standard poison. This solution when kept on ice was preserved unchanged for several days.

The experiments were made with the following animal species: man, ox, horse, goat, sheep, dog, rabbit and guinea-pig. Guided by Flexner and Noguchi's observations, we at first used only blood-cells which had been freed from serum. This was accomplished by making a 2½% suspension of the cells in 0.85% salt solution, centrifuging, decanting the fluid, and then adding anew the same amount of salt solution. This was always done twice and then a 5% suspension was made.

All the tubes of a given series contained 1 cc. of a 5% blood suspension and they were all made up to the same volume (2 to 2.5 cc.) by the addition of salt solution. The specimens were kept in the incubator at 37° C. for two hours, and then placed on ice at 6° to 8° C. until the following morning.

According to our experience there are two kinds of blood-cells so far as their behavior toward cobra venom is concerned:

- (1) Those that in themselves are dissolved by the cobra venom
- (2) Those that are affected by the cobra venom only after the addition of other substances (complements, etc.).

The following table will show the behavior of washed red blood-cells of various species toward cobra venom:

TABLE I.

Amount of Cobra Venom, cc.	1 cc. 5% Blood Suspension.							
	Guinea- pig.	Dog.	Man.	Rabbit	Horse	Ox	Sheep	Goat
1.0	complete	complete	complete	complete	complete	0	0	0
0.1	"	"	"	complete	complete			
0.05	"	"	"	almost complete	trace			
0.025	"	"	"	little	fairly trace			
0.01	"	"	"	0	0			
0.005	"	"	"			No solution		
0.0025	"	"	almost complete					
0.001	little	strong	moderate					
0.0005	trace	trace	trace					
0.0001	0	0	0					

From this, two groups of blood-cells can at once be recognized, namely, blood-cells like those of guinea-pig, dog, rabbit, man, and horse, which are dissolved by the cobra venom, and blood-cells which are not affected under these circumstances even with large amounts of the poison. The sensitive blood-cells do not all possess the same vulnerability, but manifest considerable variations, depending on the species to which they belong. This is the case with all hæmolytic poisons. Naturally besides this there are certain individual fluctuations in vulnerability. The blood-cells of the dog and the guinea-pig are the most sensitive since as a rule 0.25 cc. of a 1:10,000 dilution of the poison still produces complete solution. The blood-cells of the horse proved least sensitive, for here it required 1.0 cc. of a 1:1000 dilution of the poison to produce solution. The difference in vulnerability is therefore one of forty times.

In view of Flexner and Noguchi's experiments by which the amboceptor character of the hæmolytic portion of snake venoms was demonstrated, it seemed advisable to undertake activating experiments in those cases in which the cobra venom did not effect spontaneous solution.

It was actually very easy to produce solution by the addition of foreign sera. We shall shortly show that when the observations of Calmette<sup>1</sup> are taken into account these activities are not all due to complements. According to our conception only such substances are complements which in general are inactivated at a temperature between 52° and 60°, in some cases even somewhat higher. This corresponds to the greater or less degree of lability of the complements thus far known.

In our experiments such pure complementings were met with in the following combinations:

Horse blood.....	ox serum
Ox blood.....	guinea-pig serum
Sheep blood.....	guinea-pig serum
Rabbit blood.....	guinea-pig serum

Table II shows such an activation of the cobra venom. It also shows that the serum employed lost its complementing property by half an hour's heating to 56°.

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<sup>1</sup> A. Calmette, Sur l'action hémolytique du venin de cobra. Comptes rend. de l'Académie des Sciences, T. 134, No. 24, 1902.

TABLE II.

Amount of the Guinea pig Serum ( $\frac{1}{2}$ ) Added.	1 cc. 5% Sheep Blood *		
	a Guinea pig Serum only.	b 0.02 cc. 1% Cobra Poison + Guinea pig Serum	
		I Normal	II Heated Half an Hour at 56° C.
cc.			
0.5	little	complete	0
0.25	trace	strong	0
0.1	0	little	0
0.05	0	trace	0
0.025	0	faint trace	0
0.01	0	0	0

From these experiments it can be seen that in the cases described the cobra venom has the character of an amboceptor and that the amboceptors are activated by serum complements which possess the ordinary degree of thermolability.

We have thought it necessary to determine the mode of action of both substances according to the method used in previous studies on hæmolysis. Hence we next studied the behavior of sheep blood-cells toward the isolated cobra venom and toward the complement. So far as the behavior toward the poison alone is concerned it can be shown that this poison is bound by the sheep blood-cells although these are not by themselves dissolved by cobra venom. This confirms the statement of Flexner and Noguchi. According to our experience, however, the blood-cells possess relatively feeble binding powers, especially in dilute solutions of the poison<sup>1</sup>. On the other hand the complement alone is not at all bound by the blood-cells. This is borne out by the fact that at 0° C. sheep blood-cells are not dissolved by cobra venom + guinea-pig serum; while at 8° C. only a trace of solution occurs. If a separation experiment is made, so that amboceptor and complement are allowed to act on blood-cells at 0° C.

<sup>1</sup>The statements of Deery and Rousae (*Archiv. internat. de plasma-codynamie et de therapie*, Vol. VI, 1909) are in entire accord with the slight binding powers of red blood-cells for snake poison. They find that in the animal body also snake poison is bound very much more slowly than diphtheria or tetanus poison. Rabbits which had been intravenously injected with a fatal dose of snake venom could be saved even after ten minutes by bleeding and transfusing fresh blood, whereas with diphtheria or tetanus poison, even though the same treatment was done immediately, the fatal ending could not be averted.



after which the blood cells and fluid are separated by centrifuge, it will be found that the blood-cells have taken up a certain portion of the amboceptors, but none of the complement. These experiments would seem to prove the amboceptor character of the cobra poison, at least for the above cases, entirely according to the ideas of Flexner and Noguchi.

## II. Concerning Endocomplements.<sup>1</sup>

We shall now analyze the phenomena which we observe with those blood-cells which, like guinea-pig blood-cells, are directly dissolved by cobra poison. This solution could be explained by assuming that cobra poison, besides the amboceptors, contains true toxins which are analogous to the diphtheria toxin and exert a toxic action, i.e., effect hæmolysis, without the intervention of a complement. In that case, however, one would be compelled to assume further that only part of the species of blood-cells react to this poison. The incorrectness of this conception is readily demonstrated.

The observation was made by earlier investigators (Stephens and Myers<sup>1</sup>) that red blood-cells which are soluble in weak solutions of poison may be insoluble in stronger solutions; and the same observation was made by us on rabbit blood. This phenomenon is entirely irreconcilable with the assumption of a preformed poison, for, *ceteris paribus*, the action of this should increase with the dose. This inhibition in consequence of large doses of poison cannot be harmonized with the toxin theory.

On the contrary it indicates that we are here dealing with a phenomenon whose significance was first pointed out by M. Neisser and Wechsberg,<sup>2</sup> which consists in this, that the bactericidal action of an immune serum, provided the amount of complement remains the same, is inhibited by an excess of amboceptor.

If we assume that the red blood-cells in themselves possess a complement fitting the amboceptor of the cobra poison, an "endocomplement," we see at once that *small amounts of amboceptor effect solution, while with large doses no solution occurs owing to diversion of the complement by the amboceptors. This diversion is due to the mass action of the amboceptors present in the fluid.* This view is easily supported experimentally. If blood-cells are treated with a solution

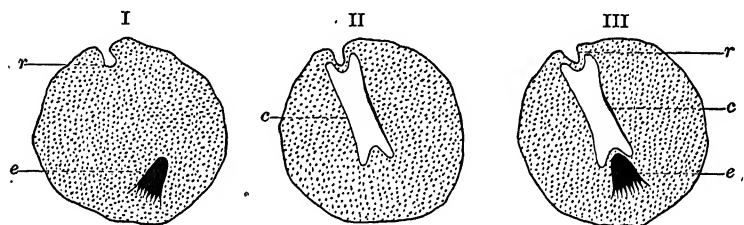
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<sup>1</sup> See also page 443.

<sup>2</sup> Journal of Pathology and Bacteriology, Vol. V, 1898.

<sup>3</sup> Münch. med. Wochenschr. 1901, No. 18. See also page 120.

of very strong snake poison, they will not be dissolved. *The mixture is now centrifuged and the sediment washed with salt solution. No solution takes place; as soon as fitting complement is added, however, solution ensues very promptly.* This shows that by the treatment with the poison the complement contained in the red blood-cells has been abstracted. The following diagram will make this clear.



- I. Blood-cell with receptor *r* and endocomplement *e*.
- II. Blood-cell after treatment with a large amount of cobra poison. The cobra amboceptor *c* has been anchored by the blood-cell receptor. The endocomplement has been abstracted from the cells by the large excess of free amboceptor.
- III. Blood-cell of stage II after the addition of complement or endocomplement *e*. The added endocomplement has combined with the cobra amboceptor *c* and can now effect solution.

The following experiment may serve as an illustration. (See Table III.)

TABLE III.

	1 cc. 5% Rabbit Blood + 1 cc. 5% Cobra Poison, Kept at 37° for Two Hours, Centrifuged and Washed. Sediments +			Controls Native Rabbit Blood + 0.15 cc. Guinea- pig Serum or 0.5 cc. Guinea- pig Endocomple- ment.
	<i>a</i> 0.85% NaCl Solution.	<i>b</i> 0.15 cc. Guinea- pig Serum.	<i>c</i> 0.5 cc. Guinea- pig Endocomple- ment (+).	
Solution effected	0	complete	complete	0

The correctness of this view can readily be shown in another way. If the blood-cells actually do contain an endocomplement, it must be possible to demonstrate this by dissolving the blood-cells in water and finding that these dissolved cells are capable of acting as complement to cobra poison for such blood-cells as are incapable of being dissolved by cobra poison alone.

As a matter of fact we have succeeded in a large number of cases in causing the solution of such cells *by the addition of laky solutions of endocomplement*.<sup>1</sup> The amount of endocomplement contained in blood-cells varies; that of human and guinea-pig blood appears to be the highest and also fairly constant.

The following table shows the combinations in which, according to our experiments, cobra poison causes solution (+) of blood-cells which are not dissolved by cobra poison alone (see Table IV).

TABLE IV.

Endocomplement of	Species of Blood.		
	Ox.	Goa.	Sheep.
Rabbit. ....	+	+	+
Man. ....	+	+	+
Dog. ....	+	+	+
Guinea-pig. ....	+	+	+
Goat. ....	- <sup>2</sup>	-	-
Ox. ....	+	-	-
Sheep. ....	- <sup>2</sup>	-	-

It is in place here to mention another fact. The deflection of the endocomplement by large quantities of poison described in the case of blood-cells vulnerable to cobra poison succeeds equally well if the experiment is made with blood-cells insensitive to cobra poison alone (ox blood) and if dissolved endocomplements (guinea-pig) are used for activation. *There is no doubt therefore that the blood-cells themselves contain complement-like substances, endocomplements.*

So far as the behavior of these endocomplements toward thermic influences is concerned, they are seen to be somewhat more resistant in general than are the complements contained in the serum, for it requires half an hour's heating to 62° C. to inactivate them (see Table V). In the light of our present knowledge, however, we probably cannot deny the complement character of these substances merely

<sup>1</sup> As a rule these endocomplement solutions were prepared by twice washing and centrifuging a certain quantity of full blood, and then filling the sediment up to a certain volume. Either the original volume or a greater or less dilution was made up depending on circumstances. They were then salted to contain 0.85% NaCl. We have designated these dilutions as  $\frac{1}{2}$ ,  $\frac{1}{3}$ ,  $\frac{1}{10}$ , etc., endocomplement.

<sup>2</sup> Even in these cases we noticed an activation with certain specimens of blood.

because of this thermostability, especially since we know that Ehrlich and Morgenroth<sup>1</sup> have described a partial complement in goat serum which was much more thermostable. According to some unpublished studies by Shiga such thermostable complements seem to take part in the bacteriolysis of anthrax bacilli by rabbit serum. The active group of conglutins and agglutinins, which, according to Ehrlich, is analogous to the zymotoxic group of complements, is still more thermostable,<sup>2</sup> for inactivation takes place only between 50 and 75° C.

From all this it follows that we must assume the blood cells which are sensitive to the above poison, to be supplied *both with receptors and complements*. Through the intervention of the anti-receptors added, the discoplasm enters into that intimate combination with the complement which is necessary in order that the latter may act.

We should like to add a few explanatory remarks to these statements, and shall begin with the *conception of complements as endocomplements*. One could, for example, assume that the endocomplements are derived not from blood-cells themselves but from the serum still adherent to these. However, we believe that the repeated washing and centrifuging has completely freed the red blood cells from serum. Guinea-pig blood-cells were washed and centrifuged eight times, yet even after that the dissolved blood-cells manifested the complement action. This excludes the possibility of the action being due to adherent serum. Another thing which speaks against this is the fact, now and then observed by us (mostly, to be sure, merely indicated) that the last decantations activated more strongly even than the first. If the washing removed adherent serum constituents, the first washings should contain more than the later ones. As a matter of fact just the reverse was found to be the case, which indicates that we are dealing with an extraction phenomenon.

In one case we even succeeded in entirely removing the endocomplement by means of salt solution. This was a suspension (5% in 0.85% salt solution) of rabbit blood, which is dissolved by cobra poison. This suspension was kept in a refrigerator for twenty-four hours and then centrifuged, when it was found that the sedimented blood-cells suspended in fresh salt solution were no

<sup>1</sup> See pages 11 et seq.

<sup>2</sup> See Bail, *Archiv für Hygiene*, Vol. XLII, 1902, also Eisenberg and Volk, *Zeitschr. f. Hygiene*, Vol. XXIV, 1902.

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TABLE V.

IN ALL CASES 0.02 CC. 1% COBRA POISON.

A.

Amount of the Endocom- plement (1/20). cc.	1 cc. 5% Ox Blood + Guinea-pig Blood Endocomplement (1/20).	
	a Normal.	b Heated to 62° for ½ Hour.
1.0	complete	0
0.75	"	0
0.5	"	0
0.25	trace	0
0.1	0	0

B.

Endocom- plement (1/10).  cc.	1 cc. 5% Goat Blood + Guinea-pig Blood Endocomplement (1/10).			
	a Normal.	Heated Half an Hour to		
		b 56° C.	c 60° C.	d 62° C.
1.0	complete	strong	trace	0
0.5	moderate	little	"	0
0.25	little	trace	0	0
0.1	faint trace	0	0	0

C.

Endocom- plement (1/10).  cc.	1 cc. 5% Sheep Blood + Guinea-pig Endocomplement (1/10).			
	a Normal.	Heated Half Hour to		
		b 56° C.	c 60° C.	d 62° C.
1.0	almost complete	moderate	trace	0
0.5	little	trace	0	0
0.25	trace	"	0	0
0.1	0	0	0	0

longer dissolved by the cobra poison, or were only very slightly dissolved. If our view was correct, the endocomplements would now be found in the decanted fluid. This proved to be the case, for the addition of suitable amounts of this fluid sufficed to cause solution of the blood-cells which were insoluble in cobra poison alone. We

were unable to obtain a like result in two similar cases. Evidently slight variations in the experiment and possibly also minute changes, and impurities leading perhaps to certain ion actions, play a rôle which it is difficult to control. We were not interested enough to follow up these relations; but we believe that had we done so we could have made the conditions more favorable for washing out the endocomplements. We merely mention this because Flexner and Noguchi state that in their experiments after repeated washings of the blood-cells all of these were found insoluble in cobra poison alone.

These authors did most of their work with snake poisons different from ours (*Crotalus adamanteus*,<sup>1</sup> *Ancistrodon contortrix*, etc.). How far this fact is responsible for the divergence cannot here be decided, nor whether the escape of the endocomplements was favored by other conditions in the experiments.<sup>1</sup>

That the endocomplements cannot be derived from the serum is also shown by the observation frequently made by us that the serum of several species of blood, whose blood-cells exhibit a plentiful supply of endocomplement, does not possess the slightest activating power, but that, on the contrary (as in the case of rabbit serum), it sometimes hinders hæmolysis of the homologous blood-cells by snake poison.

So far as the condition is concerned in which the endocomplements exist, we must assume, in those cases in which the blood-cells are directly soluble, that the endocomplement is contained free in the blood-cells. In those blood-cells, which are primarily insoluble, it will either be absent or be present in a latent form. We believe the endocomplements are absent in the goat, for in no case were the dissolved goat blood-cells able to activate cobra venom for goat blood. On the other hand, ox blood is sensitized for cobra venom by dissolved ox blood-cells, so that we shall have to assume that ox blood does not contain endocomplements in available form and that these endocomplements are changed into an active form when the cells are dissolved.

We shall reserve for subsequent study the question as to whether the endocomplements are of simple constitution or complex.

Attention is called to the fact that the existence of endocomplements furnishes another objection to Bordet's view that the

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<sup>1</sup> We shall merely say that *Daboia* poison, which through Lamb's pretty experiments has been shown to differ from cobra poison, does not dissolve rabbit blood.

antibody is only a key which makes possible the entrance of the complement into cell. For in these cases the complements which are able to destroy the blood cell are already present within the same before the antibody is anchored, and yet the blood cell is in no way injured. *The injury takes place only when a particular organic relation has been effected between complement and protoplasm by means of the antibody.*

Finally, the demonstration that the red blood cells contain complementing substances is exceedingly important in other directions. The French school in particular was inclined to refer the source of the complements exclusively to the leucocytes. We now see that the red blood cells, heretofore considered merely as concerned with the oxygen exchange, are also carriers of special complement-like substances. This confirms the view expressed by Ehrlich<sup>1</sup> in his "Selbstbeobachtungen," namely, that "the red blood discs also exercise other functions hitherto overlooked." "The red blood cells serve as *storage centres* in the sense that they temporarily take up into themselves substances characterized by the presence of hydropore groups and derived from the internal metabolism or from the food."

### III. Cobra Venom and Lecithin.

Having demonstrated that the antibody of snake poison can be complemented by easily destructible complements which may be present either in the serum or in the red blood-cells, we go on to a series of other phenomena in which activation is effected by more stable substances which are in no way related to the complements. Calmette,<sup>2</sup> in following up the work of Flexner and Noguchi, found that certain normal sera when heated to 62° C. became much better able, in conjunction with cobra poison, to cause hemolysis of the washed blood-cells. In fact it was found that fresh sera, added in large excess, can retard or even inhibit hemolysis, while these same sera when heated cause immediate solution of the blood-cells in the presence of cobra poison. From this Calmette concludes that such a blood serum must contain a natural antihemolysin which can protect the red blood-cells up to a certain point against solution by the

<sup>1</sup> In Nothnagel's *Specielle Pathologie und Therapie*, Vol. 8. Vienna, 1901.

<sup>2</sup> *ib.*

snake venom. This antihemolysin is thermolabile, being destroyed by temperatures over 56° C. The other (the activating) constituent of the serum on the contrary is thermostable, since it does not lose its activity even by heating to 80° C. Calmette therefore assumes that the alexin (our complement) takes no part in the activation, but that a particularly thermostable "substance sensibilatrice" is contained in the serum besides the thermolabile antihemolysin. By the term "substance sensibilatrice," as used in French terminology, is meant the body which we term "ambceptor." The ambceptor capable of being anchored is supposed to render the red blood cells sensitive to the attack of the alexin (complement). It is hard to see just how Calmette conceives this entire process. As we already know from the researches of Flexner and Noguchi snake venom is capable of being anchored, and from all of its properties is therefore surely a substance sensibilatrice (ambceptor). If then the substance supposed by Calmette were also a sensitizer, we should have before us something absolutely unique, namely, the combined action of two sensitizers. Unfortunately Calmette has undertaken no combining experiments and therefore has furnished no proof for his view. Our own experiments, however, speak against this assumption.

In our opinion the main reasons which led Calmette to conclude that complements play no rôle in the hemolysis by means of cobra venom are:

1. That he overlooked the endocomplements.
2. That he employed too schematic a manner of activation, namely, usually only at 62° C.

We have convinced ourselves that in suitable cases (see Table VII, case IV) a blood serum, e.g. ox serum, when fresh, dissolves the red blood-cells. If this is inactivated by heating to 56° C., the action will be found to be completely inhibited, or almost so. This same serum, however, when heated to 65° C. or higher is again able to effect hemolysis. The serum heated in this manner possesses a stronger solvent power than the fresh serum, for even fractional parts of the complete solvent dose of fresh serum suffice to cause full solution (see Table VI).

This experiment was repeated many times and proves that in this case two entirely different kinds of activations occur, namely,

1. Activation by means of complements.
2. Activation by means of substances which become manifest only through heating.



TABLE VI.  
 I. (A). Human Blood + COBRA SERUM.

A.		B.		
Amesbury No. 116 Rabbit Serum		0.02 cc. 1% Cobra Poison + Ox Serum, 1:10 Dilution.		
		Heated for One Half hour to		
		a	b	c
		Normal	56° C.	65° C.
0.5	Faint trace	complete	faint trace	complete
0.35	"	almost complete	" "	"
0.25	"	strong	"	"
0.15	"	little	"	little
0.1	"	trace	"	trace

It seemed to us that it was of the highest importance to gain a further insight into these thermostable activating substances. To begin, we found that the substance is far more stable than Calmette assumed, for activation is effected even by sera which have been cooked for hours. Thereupon we investigated a number of sera in respect to their activating power and obtained results that were little less than confusing. We found sera which activated not only in the fresh state but also after heating to 56° C. and 100° C. (No. I of Table VII). Other sera did not activate either when fresh or after heating to 56° C.; they did activate, however, when they were heated to 65° and 100° C. (No. II of Table VII). As a rule in these cases the serum heated to 100° C. proved more powerful than that heated to 65° C. A third class of sera was found which did not activate when fresh, but activated when heated to 56° C. or higher (No. III of Table VII). Finally, there is the type already mentioned, namely, a serum which activates when fresh, is made inactive by heating to 56° C. and again made active by heating to 65° (No. IV of Table VII). We have also observed sera which activate *only* when fresh and do not again acquire this property when heated to a greater or less degree (No. V of Table VII). We see therefore that we are dealing with five different combinations,<sup>1</sup> as is shown in Table VII.

<sup>1</sup> Naturally in the case of such bloods as rabbit blood, which are dissolved by cobra poison alone, only such amounts of poison have been used which by themselves are not active, but which cause hemolysis when they are combined with suitable reinforcing agents (complements, etc.).

TABLE VII.

Activating Power of the Serum.				Characteristics	
	a	b		serum	blood cell
		Heated to			
	Normal.	56° C.	65° or 100° C.		
I	+	+	+	horse	ox
				horse	goat *
				horse	horse
				man	man
				rabbit	ox
II	0	0	+	man	goat *
				man	ox
				sheep	sheep *
				rabbit	goat *
III	0	+	+	ox	ox
				sheep	ox
IV	+	0	+	guttae pig	ox
				ox	horse
V	+	0	0	guttae pig	sheep *
				guttae pig	rabbit

\* Only slight solution.

These contradictory results are not to be harmonized with Calmette's conception of a definite antibody which is destroyed at 56° C. One would have to assume that this normal antihemolysin were lacking in horse serum, for as a rule this does not become more strongly hemolytic by heating to 56° C. On the other hand in the case of a serum like No. II, which has no activating properties even when heated to 56° C., it would be necessary to believe that the activator is entirely absent. The conditions are still more complicated by the fact that one and the same serum can behave differently toward various species of blood. Thus a horse serum heated to 100° will activate cobra venom for ox blood in high dilutions (0.02 complete), whereas even in large amounts it dissolves goat blood only in comparatively slight degree (0.35 cc. moderate solution). In this case, then, the activator present is in the main one for ox blood, not for goat blood.

Believing that an insight into the nature of this maze of facts could be gained only by a thorough chemical analysis, we sought to isolate the thermostable activating substance. First we succeeded in proving that when serum is precipitated with 8 to 10 volumes of alcohol, the activating substance passes into the alcohol, while the inhibiting substance is contained in the precipitate. For if the

alcoholic extract is evaporated in vacuo and the residue dissolved in an amount of 0.85% salt solution equal to the original amount of serum, a strong activating fluid is obtained. An alcoholic extract of horse serum, when treated in this way, in contrast to the native horse serum heated to 100°C., dissolves goat blood to a high degree (0.1 cc. dissolves completely). The alcohol precipitate must therefore have contained a substance which inhibits the action of the activator, and we were actually able to demonstrate the existence of this inhibiting substance. If the precipitate is dissolved in salt water, a fluid is obtained which inhibits the hemolysis of goat blood by cobra venom and the activator derived from the alcoholic extract of horse serum. In larger, though unequal, doses it protects ox blood against solution by cobra venom and the activator. Before studying the nature of the inhibition effected by the albuminous precipitate we shall try to discover the nature of the activator. As already said, the residue obtained on evaporating the alcoholic extract was dissolved in salt water. On shaking this solution with ether, we found that the ether had taken up all of the activating substance. This proved that the activator is a substance soluble both in alcohol and ether, and one which has a wide distribution in the sera of animals. Constituents of the blood serum which are soluble in ether have long been known to us. Those mainly to be considered are cholesterol, lecithin, fats and fatty acids. After several negative trials with cholesterol we found that lecithin possesses the properties of the activator, since all blood-cells are rapidly dissolved when cobra venom and lecithin are allowed to act on them simultaneously. Not only blood-cells which are insoluble in cobra venom alone, such as goat blood-cells, but also those which are deprived of endocomplements when treated with strong solutions of poison (see § II, Endocomplements) are promptly dissolved by the lecithin. Our solution of lecithin<sup>1</sup> was made in the

<sup>1</sup> The lecithin employed by us was derived from yolk of egg and obtained from E. Merck, Darmstadt. It was a neutral mass of salve-like consistency, which was entirely precipitated from its ethereal solution by acetone (Altmann-Henriques). Even when thus purified it manifested the activating power unchanged. We reserve for further study our experiments with the pure lecithin prepared after the method of P. Bergell (Ber. der deutsch. chem. Gesellschaft, Jahrg. 33, 1900, page 2584) and with the homologues of this body. A specimen of lecithin obtained from J. D. Riedel, Berlin, corresponded exactly in its activity to Merck's lecithin. Cerebrin and Protagon, obtained through the courtesy of Prof. Kessel of Heidelberg, possess no activating power.

purest methyl alcohol, for, as we know from special experiments, this does not injure the red blood-cells even in concentration up to 4 or 10%. A 1% stock solution was diluted with 0.85% salt solution, and it was found that 0.0025 cc. to 0.0035 cc. of the 1% solution (i.e. 0.000025 g. lecithin) were sufficient to completely dissolve 1 cc. of ox or goat blood on the addition of suitable amounts of cobra poison. (See Table VIII).

TABLE VIII.

Amount of the 1% Lecithin.	0.002 cc. 1% Cobra Poison.	
	Ox Blood	Goat Blood
0.005	complete	complete
0.0035	"	"
0.0025	"	moderate
0.0015	almost complete	traces
0.001	little	0
0.00075	0	0

In what way now are we to picture the action of this lecithin? We know that lecithin is able to combine with albuminous bodies, sugars (Henriquez and Bing), etc. A threefold question had to be decided. First, whether cobra venom unites with lecithin after the fashion of an amboceptor; second, whether perhaps the snake venom had made the blood-cells sensitive to lecithin; or third, whether the reverse holds true.

A preliminary test was made to see whether lecithin and snake poison combine with one another. The method of making this experiment is relatively simple. Lecithin can easily be shaken out of its solution in salt water by means of ether. As the following experiment will show, lecithin passes into the ether in great abundance, but not completely. This behavior corresponds to a general phenomenon which is the expression of the "loi de partage." If, however, to the same amount of lecithin a suitable quantity of snake venom is added, it is found that but very little passes into the ether on shaking the ether with the mixture. Two portions each of 10 cc. were thus shaken out with ether: A, containing 2 cc. of a certain lecithin solution; B, containing besides this 1 cc. of a 1% solution of cobra venom. Previous to this both solutions were kept at 37° C. for half an hour. The ethereal extract was evaporated and the residue taken up in 10 cc. 0.85% salt solution. The action, on ox

Based on cobra venom, of the ethereal extract residues on the one hand, and of the solutions which had been shaken out on the other, is shown in Table IX.

TABLE IX.

Complete solvent dose of lecithin extract solutions with 0.1 cc. of 0.1% cobra venom—0.001 cc. corresponding to 0.025 cc. of the shaken out solution.

For 5% or Blood 0.1 cc. 0.1% Cobra Poison

Amount of A. or of B. cc.	A. Lecithin Only		B. Lecithin + Cobra Poison	
	I	II	I	II
	Ether Extract	Aqueous Portion	Ether Extract	Aqueous Portion
1.00	complete solution	complete solution	complete solution	complete solution
0.5	" "	" "	moderate	" "
0.25	" "	" "	"	" "
0.1	" "	0	"	" "
0.05	" "	"	"	" "
0.025	Traces	"	"	" "
0.015	0	"	"	0

It can be seen from the table that on the addition of snake poison to the same lecithin solution only  $\frac{1}{8}$  part of that amount of lecithin passed into the ether which passes into ether when a pure lecithin solution is shaken out. The cobra venom had therefore bound the lecithin.

The next question to determine was how the red blood-cells behaved toward cobra venom and lecithin alone and toward mixtures of these substances. In order to retard the course of the reactions as much as possible and to secure a better view of the processes we sought to create such retarding conditions by making the test with dilute solutions and at 0° C. This necessitated a preliminary quantitative determination of the effect of each factor separately. Corresponding to the slight affinity of the cobra ambceptor for the red blood cells, it was found that with suitable conditions (2 hours at 0° in dilute solutions of the poison) the ambceptor is not anchored; neither is lecithin by itself bound by the blood-cells. On the other hand blood-cells to which cobra venom + lecithin were added in suitable quantities were rapidly dissolved even at 0° C. Both components must therefore have been bound. The following table (Table X) illustrates this behavior.

TABLE X.

Complete solvent dose of cobra venom (0.1%) in the presence of 0.01 cc. lecithin  
 —0.005 cc. Complete solvent dose of lecithin in the presence of 0.1 cobra  
 venom (0.1%) —0.005 cc.

## A

Amount of Cobra Venom Added (0.1%), cc.	1 cc. 5% Ox Blood + Decreasing Amounts of Cobra Venom kept Two Hours at 0°, then Centrifuged and Washed. Thereupon 0.01 Lecithin Solution Added to	
	I. The Sediments.	II To the Decanted Fluid which had been Added to Native Ox Blood.
0.01	faint trace solution	complete solution
0.05	0	" "
0.025	0	" "
0.01	0	" "
0.005	0	almost complete
0.0025	0	"

## B

Amount of the Lecithin Solution Added, cc.	1 cc. 5% Ox Blood + Decreasing Amounts of Lecithin kept at 0° C. for Two Hours, then Centrifuged and Washed. Thereupon 0.1 cc. Cobra Venom (0.01%) Added to	
	I. The Sediments.	II To the Decanted Fluid which had been Added to Native Ox Blood
0.075	trace solution	complete solution
0.05	" "	" "
0.025	" "	" "
0.01	" "	" "
0.0075	" "	" "
0.005	" "	0

## C

Amount of Cobra Venom Added (0.1%), cc.	1 cc. 5% Ox Blood + 0.025 Lecithin Solution + Increasing Amounts of Cobra Venom, Two Hours at 0° C.		
	I. Degree of Solution Effectd.	II Heparinized Ox Blood (not centrifuged), the Sediments Washed	III Heparinized Ox Blood (not centrifuged), the Sediments Washed
0.1	complete	complete	complete
0.05	"	complete	complete
0.025	"	complete	complete
0.01	"	complete	complete
0.005	faint trace	0	complete
0.0025	0	0	moderate
0.001	0	0	0
0	0	0	0

These results can be explained only by assuming that lecithin and cobra antioceptor have combined to form what may be termed the "lecithin" of cobra poison, and that the affinity of the cobra antioceptors cytophile group is thereby increased. According to this the union with the lecithin causes the cobra poison to be more rapidly anchored than the cobra antioceptor alone. The increase of the cytophile groups affinity through the occupation of another group is perfectly conceivable chemically. An analogy frequently met with is the fact that the anchoring of the haemolytic serum antioceptors by the blood-cells usually causes an increase in the affinity of the complementophile group. Ehrlich and Sachs<sup>1</sup> have shown that the occupation of the complementophile group of serum antioceptors can cause an increase of the cytophile group's affinity, such as is presented in this case.

We therefore assume that the lecithin acts as a kind of complement since it is anchored by certain definite groupings of the poison molecule. In this way a poisonous double combination is formed of which perhaps the cholin residue constitutes the toxophore group.

There is another fact which supports the view here presented, namely, that the lecithin antioceptors effect solution of the red blood-cells even at 0° C., whereas the thermolabile complements of blood serum are anchored only at higher temperatures. Corresponding to the views formulated by Ehrlich and Marshall<sup>2</sup> for the antioceptors (polyceptors) of blood serum, we must therefore assume that the snake venom antioceptor in addition to its cytophile group possesses at least two haptophore groups, of which one as usual is able to bind complements, the other to bind lecithin. Each of these combinations by itself is *dominant*, i. e., sufficient to effect solution of the blood-cells. It is very probable that occupation of both groups increases the solvent effect.

The following experiment furnishes additional proof that the phenomena observed cannot be regarded in the light of a sensitization. The amount of lecithin required for complete haemolysis is determined in two parallel series, one on the addition of small amounts of cobra venom, the other with large amounts. It is found that far more lecithin is required for complete solution when there is a large excess of cobra venom. (See Table XI.)

<sup>1</sup> See pages 209 et seq.

<sup>2</sup> See pages 226 et seq.

TABLE XI.

Amount of Lecithin Solu- tion Added.	1 cc. 5% Ox Blood	
	0.4 cc. 5% Cobra Venom.	0.1 cc. 0.1% Cobra Venom.
cc.		
0.05	complete solution	complete solution
0.035	moderate	" "
0.025	little	" "
0.015	faint trace	" "
0.01	0	" "
0.0075	0	moderate
0.005	0	trace
0.0035	0	0

Now if the cobra venom sensitized the blood-cells for the lecithin, less lecithin would be required for solution the more cobra venom were added. As a matter of fact the reverse is the case. When we used a large excess of poison, five times as much lecithin was required for complete solution as when smaller doses were used. This is readily explained by assuming that a large excess of amboceptor causes a deflection of the lecithin, a phenomenon which we have already met with in the endocomplements.

The phenomena observed by us also serve to explain most easily the inhibiting action exerted by certain sera. As is well known, lecithin is able to combine with albuminous bodies, sugars, etc. If this union is so firm that it is not disrupted by the affinity of the cobra amboceptor, it will be impossible for the lecithin to come into action. This is the case, for example, with ox serum, which when fresh does not exert a trace of activation on goat blood, and yet the ox serum contains sufficient lecithin, as we know by examining its alcoholic extract.

Ox serum is even able to prevent hemolysis on the addition of free lecithin, the reason being evidently because it contains an excess of inhibiting substances. On heating the serum these substances lose their action to a greater or less extent, so that the serum is able when mixed with cobra venom to effect hemolysis. As already mentioned, however, the hemolytic action is usually considerably stronger when the sera are heated to 100° C. instead of only to 65° C. Perhaps this is due to substances possessing different degrees of thermolability.

In other cases only a very slight difference is to be observed



between the activating power of fresh and of heated serum. In this case evidently the fresh serum already contains free, i.e. active, lecithin, and the inhibiting substance is affected but to a slight degree by the heating. In view of all this it is certainly incorrect to speak, as Calmette<sup>1</sup> does, of a definite thermolabile antibody which is destroyed at 56° C.

It is natural to attempt a quantitative estimation of the cobra amboceptor by means of the binding of lecithin; also to think of the possibility of isolating the cobra amboceptors as lecithids. Experiments in this direction are now under way.

The results of the experiments here given furnish a further insight into the nature and mode of action of the amboceptors. The *demonstration of endocomplements*, as well as the significant fact that *a definite chemical and crystalline substance, lecithin, can in a certain sense play the rôle of complement*, would appear to be especially important for the development of our knowledge concerning poisons.

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<sup>1</sup> One might assume that the hæmolysis by cobra venom alone, ascribed in § II to the action of the endocomplements, was caused by the lecithin contained in the red blood-cells. This assumption, however, is at once excluded by the fact that the endocomplement solutions are inactivated by heating to 62° C., showing that their action has nothing to do with that of the lecithin.

## XXVIII. FURTHER STUDIES ON THE DYSENTERY BACILLUS.<sup>1</sup>

By Dr. K. SHIGA.

WHEN I discovered the dysentery bacillus in 1897 I found that although this organism apparently remains localized in the intestine and does not pass into the circulation, it nevertheless gives rise to the development of specific antibodies in the serum. This fact, made use of after the manner of the Gruber-Widal reaction, furnished me with an important aid in the diagnosis of the dysentery bacillus.

In the course of the following years the facts which I observed in connection with epidemic dysentery have been confirmed in various parts of the world,<sup>2</sup> especially since Kruse succeeded so well in his studies on this disease in Germany. To-day there is no longer any doubt concerning the identity of the bacillus isolated by Kruse with mine, even though there is still a slight divergence concerning certain morphological details. All of the important characteristics of the bacilli discovered by me, as well as their agglutination by serum of the patients, have been confirmed by Kruse. That certain slight differences in growth may occur is not at all uncommon in other bacteria, even in cholera. The question as to the presence of motility is especially hard to answer. At first I stated that my bacilli were motile; Kruse found them immotile. It is well known that it is not always easy to decide whether a bacillus is motile or not, and Kruse himself says concerning motility as a characteristic of the coli group (Flügge, Vol. II, page 361) that "one must be very careful in deciding this point, for the movements often last but a short time and are not present under all conditions of life (nutrient medium,

<sup>1</sup> Reprint from the *Zeitsch. f. Hyg. und Infectious-Krankheiten*, Vol. 41, 1902.

<sup>2</sup> Compare also the study published since this, entitled "Untersuchungen über die Ruhr," Berlin, 1902.

temperature, etc.).” In this connection I would call to mind the bacillus of erysipelas of swine, whose immotility is still questioned by many observers. I have always described the motility of my cultures as feeble, though I found it strange that I was unable at first to demonstrate flagella by staining methods. Later on, however, I succeeded in finding two terminal flagella in one préparation, and thought that this question might now be regarded as closed. To what extent this was an error I should not yet like to say, and for the present I should also not like to regard the observations of Vedder and Duval,<sup>1</sup> who found peritrichal flagella, as a confirmation of my findings.

In 1898 I immunized horses with dysentery bacilli and obtained a high-grade serum with which in 1898-1900 almost three hundred people have been treated. It therefore seemed advisable to study this dysentery serum from the standpoint of the modern theory of immunity. At the same time I was anxious by means of serum diagnosis to again prove the identity of Kruse's bacillus with mine.

The cultures employed were the following: One of my original cultures, one from Prof. Flexner, one culture of the Kruse bacillus from the Frankfurt Institute, and a Kruse bacillus from Dr. Conradi, Berlin. I may at once say that in all the various bactericidal experiments these cultures behaved exactly alike, and I shall therefore in the following speak of the dysentery bacillus as such. When I come to speak of the agglutination I shall make mention of certain variations of Flexner's bacillus from mine and Kruse's.

To begin, the bactericidal action of normal active sera was tested on the dysentery bacillus. The method employed corresponded exactly to that described by M. Neisser and Wechsberg, to whose paper I shall therefore refer.<sup>2</sup>

The amount of culture planted was always 1/500 mg. of a one-day agar culture, and in the dilution employed this was contained in 1.0 cc. salt solution. The total amount in each tube was always 2.0 cc., to which quantity three drops of bouillon were then added. The serum was allowed to act for three hours at 37° C., after which time six drops were made into agar plates. In judging the plates we did not make use of accurate counting, but always employed the

<sup>1</sup> The Etiology of Acute Dysentery in the United States. Journal of Experimental Medicine, 1902, Vol. VI., No. 2.

<sup>2</sup> See pages 120 et seq.

method of Neisser and Wechsberg, namely, approximate estimation, because only large results were regarded as conclusive. Frequently after the six drops had been taken from the tube, the residue was again placed into the incubator. In this way one often obtains valuable confirmation of the agar plates by noting whether or not there is a growth in the tubes.

The strongest bactericidal power is possessed by goat and sheep sera, but this is but slight in comparison to their action on many other species of bacteria. 0.3 cc. of these sera almost completely killed the bacteria under the conditions mentioned. Other sera are weaker, such as ox, horse, human, dog, guinea-pig, and rabbit serum. A reactivation of normal inactive sera succeeded only in the following combination: normal inactive goat serum could be completely reactivated by normal active horse serum in an amount which by itself did not kill the bacteria. These experiments showed that only a few sera could be used for reactivation (e.g. horse serum) apparently because the other sera did not contain any considerable excess of free dominant complement, or contained none at all. This was entirely confirmed by the complementing experiments which were made with a high-grade immune serum. The immune serum used was obtained from a horse which I myself had begun to immunize and which had been further immunized in the meantime. The serum was sent to me from Japan with the addition of 0.5% carbolic. In the small amounts in which the serum was used, this addition in no way disturbed the bactericidal experiments, as was shown by control tests. The first experiments undertaken with the completion by means of active horse serum resulted negatively in so far as any destructive action was concerned. This was soon found to be due to the phenomenon of complement deflection described by Neisser and Wechsberg; for when smaller and still smaller doses of the immune serum were employed the destructive action became more and more marked. Table I, in which column A gives the result of the plate tests, and B that of the test-tube experiment made at the same time, shows the destructive action as well as the phenomenon of complement deflection.

From this it is seen that even 0.0025 and 0.0005 cc. still have a distinct bactericidal action. This result was obtained a great many times, with various strains, in almost the same manner.

Besides the horse serum only one other serum could be used

for complementing the immune serum, namely, active human serum. Table I shows an experiment with this serum.

TABLE I.

Inactive Dysentery Serum.	Active Human Serum.	Dysentery Culture.	A. No. of Colonies on the Plate.	B. Growth in the Tubes.
0.01	0.3	10 cc. (1/500 mg.)	✓	+
0.0075	0.3	"	✓	+
0.005	0.3	"	✓	+
0.0025	0.3	"	almost 0	—
0.001	0.3	"	0	—
0.00075	0.3	"	almost 0	—
0.0005	0.3	"	about 50	—
0.00025	0.3	"	" 100	+
0.0001	0.3	"	" 1000	+
0.000075	0.3	"	several thous.	+
0.00005	0.3	"	✓	+
Control	0.3	10 cc. (1/500 mg.)	several thous.	+
	"	"	✓	+
	0.1	"	0	—
	0.3	"	0	—

TABLE II.

Inactive Dysentery Serum.	Active Human Serum.	Dysentery Culture.	No. of Colonies on the Plate.
0.01	0.3	10 cc. (1/500 mg.)	✓
0.003	0.3	"	✓
0.001	0.3	"	✓
0.0003	0.3	"	few
0.0001	0.3	"	0
0.00003	0.3	"	about 100
0.00001	0.3	"	" 1000
Control	0.3	10 cc. (1/500 mg.)	✓
	"	"	✓
	0.1	"	0
	0.3	"	0

Up to the present time I have tested the serum of six individuals and found it active in five cases (four times in placental serum and once adult serum); only once, in the case of a nephritis patient, was the fresh serum ineffective for complementing. It may be mentioned that one of these sera was my own, and this was considerably stronger than the rest. Whether this property has any connection with an active immunization which I underwent some four years previously I shall leave undecided.

I believe this demonstrates that the horse immune serum employed by me for therapeutic purposes, meets the requirements which are nowadays to be demanded of a bactericidal immune serum, namely, (1) that it be high grade, and (2) that it find a fitting complement in normal human serum. This is the first serum employed in human therapy which fulfils the conditions laid down by Ehrlich in his Croonian Lecture, 1900. The excellent curative results obtained by me in Japan<sup>1</sup> furnish abundant confirmation of the correctness of Ehrlich's views.

As already mentioned, the phenomenon of deflection of complement could be demonstrated very prettily with the complement of this active horse serum. Since this deflection is primarily dependent on the amount of immune body present, it may perhaps be possible to employ the degree of deflection as a measure of the titer of a serum. Some experiments in this direction which I have undertaken at the suggestion of Prof. M. Neisser have not yet been concluded.

I have already stated that the other active sera (e.g. goat serum, etc.) could not be used for complementing the dysentery immune serum, although in themselves they were bactericidal. But for this immune serum the phenomenon of complement deflection can be demonstrated very nicely with these sera also. (See Table III.)

TABLE III.

Dysentery Immune Serum, cc.	Active Goat Serum, cc.	Dysentery Cultures	No. of Colonies on a Plate
0.1	0.3	1/500 mg.	0
0.03	0.3	"	0
0.01	0.3	"	0
0.003	0.3	"	0
0.001	0.3	"	0
Controls	0.3	1/500 mg.	0
	0.1	"	0
	0.3	"	0

Perhaps also this method of testing is available for determining the grade of bactericidal sera.

Furthermore by means of an absorption test analogous to the experiments of A. Lipstein<sup>2</sup> I have convinced myself that the deflec-

<sup>1</sup> Deutsche med. Wochenschrift, 1901, Nos. 43-45.

<sup>2</sup> See pages 132 et seq.

tion of complement described is actually due to an excess of immune body and not, for example, to the presence of an anticomplement.

Prof. Neisser and I thought that this phenomenon of complement deflection could be utilized in another direction. Ehrlich and his pupils, it will be remembered, have demonstrated the existence of a plurality of complements. In view of this it was conceivable that following a large addition of inactive immune serum to a normal serum bactericidal per se, only that complement would be deflected which is able to complement the immune serum, while the remaining complements were left unaffected. From this it would follow that the normal active serum in question would in the main have lost only this one bactericidal action, while it still retained almost all the others. One would thus have a serum which had lost a bactericidal action chiefly for that bacterium whose immune body has been added in excess; that is to say, a *truly specific nutrient medium*. Proceeding from these considerations we first infected a normal stool with a small quantity of dysentery bacilli. To small amounts of this infected stool 20 cc. normal active goat serum and 0.2 cc. inactive immune serum were added and the mixture kept in the thermostat. At the end of three hours six drops of this mixture were added to a second tube containing 20 cc. normal active goat serum and 0.2 inactive immune serum. Agar plates were made (1) from the original infected stool, (2) from the first tube; (3) and from the second tube after it also had been kept at 37° C. for three hours. A great many tests showed that a *specific enriching in dysentery bacilli* takes place, so that when the first plate shows only a few scattered colonies of dysentery bacilli, Plates II and III show numerous colonies. In one case we even succeeded in finding dysentery bacilli in Plates II and III, although none had been found on Plate I. It may be mentioned that we used the agar medium recommended by v. Drigalski and Conrad<sup>1</sup> for the diagnosis of typhoid bacilli, and found it of great advantage. The method just described for enriching cultures may perhaps be extended and perfected.

*Proagglutinoid.*

As a result of the brilliant investigations of Bail<sup>1</sup> on the one hand and of Eisenberg and Volk<sup>2</sup> on the other, two new phenomena have been described as occurring in the agglutination reaction, phenomena which are of great importance in the study of agglutinins. Bail first showed that typhoid bacilli which had been added to an inactivated (by heat) agglutinin and then centrifuged could not longer be agglutinated by the addition of active agglutinin. The study of Eisenberg and Volk described an irregularity occurring in a series of agglutinations which manifested itself in this, that the tubes containing the largest amount of agglutinin showed only feeble agglutination or none at all, while the tubes containing less agglutinin showed strong agglutination.<sup>3</sup> Bail was of the opinion that the phenomenon observed by him was due to the interaction of two components (corresponding to amboceptor and complement), and he supported this with several reactivating experiments. Eisenberg and Volk explained the irregular course of the agglutination by the presence of agglutinoids, a view in which I fully agree.

Following Ehrlich's nomenclature I should, however, like to term these agglutinoids<sup>4</sup> *proagglutinoids*, for we are dealing with the action of substances which arise from the agglutinins as a result of external influences. Furthermore the proagglutinoids possess a higher affinity for the bacilli than the unchanged agglutinin, and they have lost that group which is the real carrier of the agglutinating action, while the other group, which effects the combination with the bacteria, is left intact.

<sup>1</sup> Archiv. f. Hygiene, 1902, Vol. XLIII.

<sup>2</sup> Zeitschr. f. Hygiene, 1902, Vol. XI.

<sup>3</sup> This paradoxical phenomenon is mentioned by Asakawa in a report from the Institute for Infectious Diseases, Tokio (Sept., 1901), and is termed by him a "reversely behaving phenomenon" ("ein umgekehrt sich verhaltendes Phänomen").

<sup>4</sup> Since the conclusion of these experiments two new studies have appeared on precipitoids. R. Kraus (Centralblatt für Bakteriologie 1902, Vol. XXXII, No. 1), v. Pirquet and Eisenberg (Extrait d. Bull. d. l'Académie des sciences de Cracovie, also Centralblatt f. Bakteriologie, 1902, Vol. XXXI, No. 15); also Wiener (Klin. Wochenschr. 1901, Über Precipitoide). The authors arrive at the same results as have been described for agglutination. Their experiments for demonstrating these precipitoids are similar to mine for the proagglutinoids.



From the large number of experiments which I have made with dysentery and typhoid bacilli I have selected only those which may serve to demonstrate my point. Using the dysentery immune serum described above I found it easy to demonstrate the Eisenberg-Volk phenomenon both with my original dysentery culture and with a Kruse culture. The method was as follows: An agar culture was suspended in 10 cc. of an 0.85% salt solution. At first this was used in the living state, later on, after it had been found that there is no difference in the action of living and dead culture, the culture was used with the addition of 0.02 cc. formalin (10%, 1). One cubic centimeter of this suspension was put into each tube, and decreasing amounts of the immune serum (2  $\mu$ l., 2  $\mu$ l., 2  $\mu$ l., etc., usually up to 27  $\mu$ l.) added, the total volume in each of the tubes being 2 cc. The tubes were then kept in the thermostat at 37° C. and inspected at the end of 2, 5, and 24 hours, both with the naked eye and with a magnifying glass. The results were noted as follows:

- no agglutination;
- ± trace agglutination;
- \* microscopically distinct but feeble;
- ++ very distinct;
- + + + entirely clear fluid with an agglutinated sediment.

TABLE IV.

Dilutions of the Dysentery Antisera	Two Hours.	Five Hours.	Twenty-four Hours.
1:10	—	—	1
1:20	—	±	± +
1:40	±	±	± + +
1:80	±	± +	± + +
1:160	±	±	± + +
1:320	—	+	± + +
1:640	—	±	± +
1:1280	—	—	±
1:2560	—	—	—
1:5120	—	—	—

The objection was made that the agglutination was hindered in the low dilutions by the large amount of serum present in the tubes. This was met by a corresponding addition of normal serum, and of other fluids (gelatine, mucilage, etc.) to the other dilutions. In the old dysentery serum the question as to the development of the

proagglutinoïd from the agglutinin could only be answered by showing that the amount of proagglutinoïd already present in this serum could be increased by heating, by continued exposure to light, or by the addition of chloroform. See Table V.

TABLE V.

Dilution of the Dysentery Serum.	The Serum Exposed to Light for 17 Days.			The Serum Heated to 60° C. for One Hour.			The Serum Shaken up with Chloroform.		
	2 Hrs.	5 Hrs.	24 Hrs.	2 Hrs.	5 Hrs.	24 Hrs.	2 Hrs.	5 Hrs.	24 Hrs.
1:10	—	—	—	—	—	—	—	—	—
1:20	—	—	—	—	—	—	—	—	—
1:40	—	—	—	—	—	—	—	—	—
1:80	+	+	+	—	—	+	—	—	+
1:160	+	+	+	—	+	+	—	—	+
1:320	+	+	+	—	+	+	—	—	+
1:640	+	+	+	—	—	+	—	—	+
1:1280	—	—	—	—	—	—	—	—	—
1:2560	—	—	—	—	—	—	—	—	—
1:5120	—	—	—	—	—	—	—	—	—

The development of the proagglutinoïd from the agglutinin was still more distinct in a fresh typhoid immune serum (goats). This serum, which had shown no zone of proagglutinoïd, showed a distinct zone after being heated twice to 60° for four hours.

By this experiment the higher affinity of the proagglutinoïd is already demonstrated. It can, however, be confirmed by other experiments. By shaking the dysentery serum with chloroform, it was possible to effect almost a complete transformation of agglutination into proagglutinoïd so that the serum hardly agglutinated in any dilution. When to a dose of the unchanged dysentery serum, sufficient by itself to effect agglutination, I added decreasing amounts of the serum treated with chloroform, no agglutination was obtained in the dilutions up to 1:160. (Control tests with chloroformed normal serum were invariably made.) The same result could be obtained with dysentery serum that had been heated. Dysentery serum heated for 3 hours to 65° C. was able in dilutions of 1:10 to 1:320 to prevent agglutination by such a dose of the unchanged dysentery serum which by itself would have sufficed to agglutinate 1:160. (See Table VI.)

Finally it remained to prove that the proagglutinoïd had really been anchored by the bacteria, i.e., that the agglutinable group of the bacilli had been blocked. This was readily accomplished by

centrifuging, washing the bacilli from those tubes in which no agglutination had occurred, and adding to them a dose of agglutinin which by itself would suffice for agglutination. The result was that these bacilli always showed themselves to be no longer agglutinable, see (Table VII).

TABLE VI

Dysentery Bacilli 1000000, 1 cc.	Dysentery Bacilli, Heated at 57°C. for Three Hours	Proagglutinin of Dysentery Culture	2 Hours.	5 Hours.
0.1 cc.	1:10	1:0.001	1:00 cc.	—
"	1:20	"	"	—
"	1:40	"	"	—
"	1:80	"	"	—
"	1:160	"	"	—
"	1:320	"	"	—
"	1:640	"	"	1
"	1:1280	"	"	3
"	1:2560	"	"	4.5
"	1:5120	"	"	6.5
Control 0.1 cc.	Salt solution 1000	1:0 cc.	—	—

One other point may be mentioned. In the experiments thus far described the quantity of bacteria was the same in all the tubes, (See above.) However, if the amount was greatly increased, other phenomena were observed. Table VIII shows that the zone of proagglutinoid disappears entirely if a sufficiently large quantity of bacteria are employed.

The explanation of this phenomenon is not difficult if we bear in mind the experiments of M. Nesser and Lubowsky<sup>1</sup> on the one hand and those of Florenberg and Volk on the other. The experiments of the latter show without doubt that typhoid bacilli, for example, are able to anchor a far greater quantity of agglutinin than is required for their agglutination. One may therefore assume that the dysentery bacillus also possesses a large number of receptors which are able to unite with, i.e., *anchor*, the proagglutinoid. The occupation of only a few of these many receptors by the active agglutinin is apparently sufficient, however, to *agglutinate* the dysentery bacillus. Hence if we add comparatively few dysentery bacilli to a serum which contains much proagglutinoid and little agglutinin, a large number of receptors of the bacilli will be occupied by proagglutinoid. If, on the contrary,

<sup>1</sup> See pages 146 et seq.

TABLE VII.

A.

Dilution of the Dysentery Serum.	24 Hrs.		To the Residue the Dysentery Serum (1:160) is Added.	2 Hrs.	5 Hrs.	Remarks.
1:10	—	Thereupon centrifuged	2.0 cc.			Not tested the second time because of the primary agglutination
1:20	—		"			
1:40	+					
1:80	++					
1:160	+++					
1:320	+++					
1:640	++					
1:1280	+					
1:2560	—		Control 2.0 cc. dysentery bacilli			

B.

Dilution of the Serum Heated to 65° for 3 Hours.	5 Hours.	24 Hours.		The Dysentery Serum (1:160) Added to the Residue.	2 Hours.	5 Hours.
1:10	—	—	Then centrifuged	2.0 cc.		
1:20	—	—		"		
1:40	—	—		"		
1:80	—	—		"		
1:160	—	—		"		++
1:320	—	—		"	+	+++
1:640	—	—		"	+	+++
1:1280	—	—		"	+	+++
1:2560	—	—		Control 2.0 cc. dysentery bacilli	+	+++

TABLE VIII.

Dilution of the Dysentery Serum.	Normal Suspension of Dysentery Bacilli.			Five Times as Strong a Suspension of Dysentery Bacilli.		
	2 Hours.	5 Hours.	24 Hours.	2 Hours.	5 Hours.	24 Hours.
1:10	—	—	—	—	++	+++
1:20	—	±	—	—	++	+++
1:40	±	+	++	—	++	+++
1:80	±	+	+++	—	++	+++
1:160	±	+	+++	—	+	+++
1:320	±	+	+++	—	—	+++
1:640	—	±	++	—	—	++
1:1280	—	—	++	—	—	++
1:2560	—	—	++	—	—	++
1:5120	—	—	++	—	—	++

we add a large quantity of bacteria to the same amount of serum, the proagglutinin will not suffice to occupy all the receptors and some agglutinin will be enabled to combine with the bacteria. This, however, results in agglutination.

As already mentioned, my original culture proved entirely identical with the Kruse culture so far as the zone of proagglutinin was concerned. The Flexner culture, on the contrary, behaved differently, for, although it was agglutinated in the same degree by the immune serum, the zone of proagglutinin was entirely absent. This is well shown in the following table.

TABLE IX.

Dilution of the Dysentery Serum.	2 Hours.	5 Hours.	24 Hours.
1:10	+++	+++	+++
1:20	++	+++	+++
1:40	++	+++	+++
1:80	+	+++	+++
1:160	±	++	++
1:320	—	+	+
1:640	—	+	+
1:1280	—	±	+
1:2560	—	—	—
1:5120	—	—	—

Absorption tests, which were then made, showed that the Kruse bacillus when added to my immune serum completely abstracted the agglutinin and proagglutinin for this strain, while the agglutinin for the Flexner strain was abstracted to only a slight degree. Conversely, when the Flexner bacillus was added to my immune serum and the mixture centrifuged it was found that the agglutinin for Flexner's bacilli had been completely absorbed, but only a small part of the agglutinin and proagglutinin for the Kruse strain.

We shall therefore have to assume that my original strain corresponds completely to the Kruse strain so far as the receptor apparatus is concerned, while both these strains possess certain receptors identical with those of Flexner's strain, and others which differ from them. We may furthermore assume that the serum with which these experiments were made was obtained by immunizing not only with my original strain, but that in the course of years various other strains had been used for immunization. In this way agglutinins of various kinds were developed, and these, of course, also fitted strains with

a somewhat different receptor apparatus. It may be remarked that the receptor apparatus of the bacteria need not permanently remain the same qualitatively and quantitatively, as is well shown by some experiments of mine in which I succeeded in producing a change in these properties by means of cultivation. Thus after having grown Kruse's bacilli on sterile milk<sup>1</sup> ten consecutive times (always transplanting on the second day), and finally transplanted it to agar, it was found that this milk strain no longer showed the zone of the proagglutinoid reaction.

On making mutual absorption tests it was seen that the organism was no longer like the original Kruse strain but entirely like that of Flexner. That is to say, this cultivation on milk had effected a gradual change in the Kruse strain which manifested itself in the changed proagglutinoid zone of the absorption power. (See Table X.)

It remains for further experiments in this direction to see whether I shall succeed in cultivating the Milk-Kruse strain back to the original Kruse strain, or in changing the Flexner strain into the Kruse strain. Thus far the Flexner strain, as well as the Flexner strain altered by cultivation, have preserved their properties for months.

#### *Résumé.*

1. In the bactericidal tests, as well as in agglutination reactions, my original dysentery strain from Japan proved entirely identical with the two Kruse cultures. Since these are the most refined methods at present at our disposal, there can be no doubt as to the identity of my original cultures of 1897 with Kruse's bacillus of 1900.

2. The dysentery immune serum derived from a horse and employed by me for therapeutic purposes in 1898-1900 is of very high grade and

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<sup>1</sup> This method of cultivation was really made because of the statement of Celli ("Zur Aetiologie der Dysenterie, v. Leydens Feestschrift") that my bacillus would also coagulate milk like the bacillus found by him, if it was transplanted 8-10 times on alkaline milk. The result of my experiment was absolutely different, for neither my original strain, nor the strain of Kruse, nor that of Flexner coagulated milk when the cultures were grown on milk ten consecutive times, provided care was taken to protect the milk from contamination. I had already tested Celli's bacillus in Japan and found that it produced a considerable amount of gas and coagulated milk, whereas my bacillus does not do this. In view of this and of the further fact that Celli's bacillus does not agglutinate with the immune serum produced by means of my bacillus, I conclude that these two organisms are entirely distinct from one another—a view which I have already expressed in a previous communication.

is the first of such sera whose complementability by human serum has been proved.

TABLE X.

Dilution of the Agglo- minating Serum	Normal Culture			First Generation of Milk Culture			Fourth Generation of Milk Culture		
	2 Hrs.	5 Hrs.	24 Hrs.	2 Hrs.	5 Hrs.	24 Hrs.	2 Hrs.	5 Hrs.	24 Hrs.
1:10			+		+	+	+	+	+
1:20		+	++	+	+	+	+	+	++
1:40	+	+	+++	+	++	+++	+	++	+++
1:80	+	+	+++	+	+++	+++	+	+++	+++
1:160	+	+	+++	+	+++	+++	+	+++	+++
1:320		+	+++	+	+++	+++	+	+++	+++
1:640		+	+++		+++	+++	+	+++	+++
1:1280			+		+	+		+	++
1:2560									
1:5120									

Dilution of the Agglo- minating Serum	Sixth Generation of Milk Culture			Eighth Generation of Milk Culture			Tenth Generation of Milk Culture		
	2 Hrs.	5 Hrs.	24 Hrs.	2 Hrs.	5 Hrs.	24 Hrs.	2 Hrs.	5 Hrs.	24 Hrs.
1:10	+	+++	+++	+	+++	+++	+	+++	+++
1:20	+	+++	+++	+	+++	+++	+	+++	+++
1:40	+++	+++	+++	+++	+++	+++	+++	+++	+++
1:80	+++	+++	+++	+	+++	+++	+++	+++	+++
1:160	+	+++	+++	+	+++	+++	+	+++	+++
1:320	+	+++	+++	+	+++	+++	+	+	+++
1:640	+	+	+++	+	+	+	+	+	+
1:1280		+	+++		+	+		+	+
1:2560									
1:5120									

3. The deflection of complement of Neisser-Wechsberg could very readily be demonstrated with this serum and pointed the way for a new method of specifically enriching bacterial cultures in mixtures.

4. The change of the agglutinin into a proagglutinoïd succeeded both in dysentery serum and typhoid serum.

5. Various strains may possess a somewhat different receptor apparatus. By means of continued culture on milk a certain change in the behavior of the receptor apparatus of dysentery bacilli could be effected.

In conclusion, I wish to express my thanks to Prof. Ehrlich and Prof. M. Neisser for aiding me in this study.

## XXIX. METHODS OF STUDYING HEMOLYSINS.

By Dr. J. MORGENROTH, Member of the Institute

THE object of the following article is to give a brief outline of the principles governing the technique of hemolytic experiments. It may be taken for granted that the methods employed in the experiments already described will be applicable to many problems of hemolysis still to be studied and to many questions concerning bacteriolysons and cytotoxins. In view of this a systematic treatise on methods will prove of considerable value, especially to one who uses these methods only occasionally. In those cases where a particular technique has been sufficiently described in the previous papers, I have contented myself with merely giving the reference to this paper.

Aside, however, from this practical object, a general survey of the subject is to be given which will show how a system of technique, intelligently built up on a comprehensive theory, has made it possible to push our analytical inquiries into a department of science which formerly constituted a sealed book to the ordinary methods of chemistry. Disregard of these newer methods has invariably led to obscurity and error, as we have been able to show on several occasions<sup>1</sup>; and in the future, even if refined *chemical* methods can successfully be introduced into this domain, the general method of analysis here outlined will always form the basis of this study. According to our experience the study of hemolysins will be much simplified by attention to a number of technical details which are described in this article.

### I. Collecting and Preserving the Blood and Blood Serum.

We shall begin with some remarks on the collection and preservation of the blood and serum required for these experiments.

As a general rule for hemolytic experiments it is not necessary

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<sup>1</sup> See, for example, pages 181 et seq.; 241 et seq.; 283 et seq., etc.



to observe aseptic precautions; usually all that is required is to collect the blood in dry sterile vessels, avoiding contamination with dirt, etc. Hence the troublesome method of collecting blood from the carotid of the animals will only then be undertaken if for some reason asepsis is necessary or a large yield of blood is required. In the latter case the yield of blood can be considerably increased toward the end of exsanguination by rhythmic compression of the cardiac region. With goats, sheep, etc., the blood can easily be obtained without any previous dissection by means of a suitable canula thrust through the skin directly into the jugular vein which has been distended by compression on the cardiac side. This is the method commonly employed in obtaining the therapeutic sera from horses. In this way small amounts of blood can be drawn from the animals a great many times. Smaller animals, such as dogs, rabbits, guinea-pigs, and rats, are most readily bled by anæsthetizing them, dissecting off the skin of the thigh and then with one stroke cutting both the femoral artery and vein. From rabbits small amounts of blood are easily obtained by incising the ear with a scissors or by means of a hypodermic needle introduced into the marginal ear vein. Small amounts of blood can be obtained from birds from the large wing vein; in the case of geese and ducks the web of the foot can be incised.

For purposes of obtaining serum the blood is collected in cylindrical vessels and allowed to coagulate spontaneously. It is kept in the refrigerator until the serum has separated. Several hours after collecting the blood, it is well to loosen the clot from the sides of the tube by means of a glass rod or spatula, for if this is not done the serum may not separate. Small amounts of blood are best allowed to clot in cylindrical glasses or tubes placed slantingly. After clotting has occurred the vessel is placed upright. The serum which separates will then flow to the bottom and can be poured off the next day. If the serum is clouded with blood-cells, these are to be removed as soon as possible.<sup>1</sup>

When the serum is poured off the first time the vessel containing

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<sup>1</sup> An excellent centrifuge with a capacity up to 200 cc., but which can also be had for larger quantities, is that made by Runne, the mechanic in Heidelberg University. This machine is made either for water or electric power, and runs exceedingly smoothly. For centrifuging smaller quantities of fluid, and especially for sedimenting blood-cells from *dilute* blood suspensions, the hand centrifuge designed by Steenbeck-Litten, and made by F. and M. Lautenschläger in Berlin, is excellent.

the clot can be kept on ice for 24 hours longer. In that way a further yield is obtained.

In order to obtain serum *immediately* the blood is defibrinated by whipping it with a stick of wood or by shaking it in a bottle containing some glass beads, or still better a little mass of dry sterilized iron turnings. After the blood is defibrinated it is centrifuged and the serum carefully separated by means of a pipette. It is well to fasten a long rubber tube to the upper end of the pipette and have an assistant suck while one watches the point of the pipette.

So far as concerns preservation of the serum it may be said that our present experiences are not yet sufficient to permit us to formulate safe rules having general applicability. It is not only necessary to prevent putrefaction, but also to preserve intact a large number of most unstable substances, the conditions necessary for whose existence are, in part, evidently very narrowly limited. Hence for the present it may be put down as a rule that in all important primary determinations only *very fresh serum* should be employed. This applies above all to the study of the complements. *Negative* results with sera which have been kept several days and which have been exposed to any kind of thermic or chemic influence, are particularly unreliable. Hence it is necessary that those properties of a serum which one purposes to study should be examined *before* the serum is preserved, so that secondary changes can then be controlled at any time.

The easiest substances to preserve are the antitoxins, anticomplements, antiamboceptors and the majority of artificially-produced amboceptors. By the addition of carbonic acid, Pfeiffer<sup>1</sup> has succeeded in keeping a cholera immune serum derived from a goat for five years without decrease in strength. We have preserved hæmolytic amboceptors for a long time without any addition, by keeping the sera in an ice-chest at 8° C. The development of bacteria is usually prevented by heating the serum in the test-tubes stoppered with cotton plugs to 57° for half an hour. In this way the serum is both inactivated and sterilized. So far as our experience goes the anticomplements and antiamboceptors can be preserved in the refrigerator like the amboceptors. Drying the serum over sulphuric acid or over anhydrous phosphoric acid in vacuum can also be used for these substances.

Of all the substances here concerned the complements are by far the most labile; whenever possible, therefore, *fresh* serum is used

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<sup>1</sup> See Mertens, Deutsche med. Wochensch. 1901, No. 24.

for activation. Most of the complements will keep unchanged for a number of days provided the serum is kept on ice. But this does not preclude unpleasant surprises, diminutions in the complementing power often occurring to a high degree without any assignable cause. According to our experience the complements of guinea-pig serum and goat serum are relatively stable. The least reliable in this respect is horse serum, whose complementing powers are often partially or completely destroyed within twenty-four hours. The complements also suffer when the serum is dried; at least that has been the case in our rather limited experience.

The best method of preserving the complements for a long time, and the one almost always reliable in all cases, consists in freezing the serum at  $-10^{\circ}$  to  $-15^{\circ}$  C. This method has been employed in the Institute for a long time. The serum is bottled in little vials, which are then kept in a freezing apparatus or in a well insulated freezing mixture of ice and salt, each vial being thawed out as needed. This procedure is at present the only one which is of general applicability and which preserves the various constituents of the serum for a long time.

The blood used for the hemolytic tests is defibrinated by one of the methods above mentioned. In special cases, instead of defibrinating, one can prevent coagulation by precipitating the lime salts. This is done by allowing the blood to flow into salt solution to which citrate of soda has been added, as was recommended by Ehrlich.<sup>1</sup> For the majority of experiments the blood is diluted with physiological salt solution. If for any reason one wishes to remove the serum, the blood is separated by centrifuge and the suspending fluid renewed several times. As a rule blood which has been kept on ice for two days can still be used.

It should also be mentioned that a suitable salt solution should be employed for each species of blood. For the blood-cells of most mammals a feebly hypertonic solution of NaCl 0.85% is best adapted. In 0.85% salt solution dog and horse blood frequently shows a slight amount of spontaneous hemolysis which can often be prevented by using a somewhat higher concentration (0.95%) of the salt. As a rule strongly hypertonic solutions of salt are to be avoided because the increased contents of salt markedly inhibit hemolysis.<sup>2</sup>

<sup>1</sup> Ehrlich, *Fortschritte der Medizin*, 1897, No. 2.

<sup>2</sup> S. Markl, *Zeitsch. f. Hygiene*, Vol. 39.

## II. The Method of Making Hæmolytic Experiments. General Considerations.

With a little practice the quantitative estimation of hæmolysis proves very simple. The two fundamental points, entire hæmolysis (complete), and no hæmolysis whatever (0), are usually very readily recognized. By "trace" we mean the occurrence of a faint zone of solution observed just above the cells by gently agitating the test-tube. The estimation of complete hæmolysis only then offers difficulties if considerable agglutination has occurred, so that the fluid when shaken is clouded by the clumped stromata. Such cases in themselves are poorly adapted for quantitative studies because at times the rapid agglutination may purely mechanically prevent the escape of the hæmoglobin and so simulate an absence of hæmolysis.

In this respect according to our experiences the greatest difficulties are presented by dog blood-cells and the specific immune sera (derived from goats) against these. This is still more the case in such sera derived from rabbits. It often happens, before even a trace of hæmolysis has occurred, that the dog blood-cells are agglutinated and fall to the bottom of the test-tube. Goose blood and specific immune serum behave similarly. In these cases it is necessary by means of frequent shaking to separate the agglutinated blood-cells so that the hæmoglobin is given chance to escape.

In those cases in which the usual method of describing the degree of solution does not suffice, and accurate quantitative determinations of the amount of blood-cells dissolved are desired, one makes use of a colorimetric procedure devised by Madsen in which a color comparison is always made by dissolving blood-cells in water.<sup>1</sup>

Agglutination is usually easily recognized on shaking up the sedimented blood-cells. It becomes very evident when the specimens of blood are shaken and one then compares the rapidity with which the blood-cells settle to the bottom. This is always greater with agglutinated blood-cells.

In general a 5% suspension of the blood-cells in 0.85% salt solution has proven best adapted for hæmolytic experiments. 1 to 2 cc. of such a mixture in each test-tube is sufficient for most tests. When material is scanty one can use amounts very much smaller, though usually this will be at the expense of accuracy. In this case, of

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<sup>1</sup> See Madsen, *Zeitschrift für Hygiene*, Vol. 32, 1899.

course, the test is made in very narrow test-tubes.<sup>1</sup> The serum to be tested is added to the various tubes in decreasing amounts. The volume of fluid should be made the same in all the tubes by the addition of salt solution, for the total amount of fluid present may influence the course of hemolysis. We usually keep the tubes in a thermostat at 37° C. for two hours, frequently shaking them if necessary. They are then kept in the refrigerator at 8° C. overnight, which allows the intact blood-cells to settle. In the cases thus far examined by us this method has always sufficed to produce the maximum amount of hemolysis, though, of course, in a given case it may have to be modified to suit the circumstances.

It should be mentioned that in testing any substances for hemolytic action, the blood-cells must always be freed from serum by repeated washing, for the serum may in some instances (e.g. with solanin) give rise to a marked inhibitory action and so lead to errors.

### III. The Technique of Immunization.

So far as the production of *hemolytic antibodies* by means of immunization is concerned, only a few very general rules can be given, for thus far sufficient systematic investigations have not been made to determine the optimal conditions in any one direction. In immunization one always selects such animals whose serum by itself is not at all or but slightly hemolytic for the blood employed, for then the development of a hemolysin is most readily determined and the normal serum of this species always furnishes an ideal complement. If animals are immunized whose serum by itself already acts hemolytically on the blood used, it is necessary to make an exact preliminary determination of the hemolytic power of the normal serum, and also to make a simultaneous control with normal serum, when making the hemolytic experiments.

In some instances it may be necessary to subject the blood to a preparatory treatment, for the purpose of removing the serum more or less completely. This is done by means of the centrifuge and is required especially in those cases in which intravenous injections are made, or if large amounts of a blood are employed whose serum

<sup>1</sup> In certain cases the employment of very high columns of blood is indicated, for in that case the development of zones (colorless - feebly red - strongly red) permits of a very accurate estimation of the period of incubation of the poison, or of the different vulnerability of the blood-cells. See also Madsen, l.c.

is highly toxic for the animal injected. If, for example, a rabbit is injected intravenously with 10 cc. of dog blood whose serum has not previously been removed, the animal will die acutely. By previously heating the serum one also obviates the reactive production of serum coagulins and anticomplements, both of which can at times hinder the estimation of hæmolysis. A general rule as to which mode of injection is to be chosen for immunization cannot be laid down. Larger laboratory animals are usually injected subcutaneously; goats usually bear intraperitoneal injections very well. This mode of injection, using blood-cells which have previously been dissolved with water, is used especially when a particularly marked "ictus immunisatorius" is desired, as, for example, in the production of isolysins. Birds are injected into the large pectoral muscles or intraperitoneally. For rabbits and guinea-pigs the intraperitoneal injections are well adapted, since, if the material is not positively sterile, secondary injections (which in subcutaneous inoculations often lead to troublesome abscesses, especially in the rabbit) are most readily avoided. Injuries to the intestine are best avoided by holding the animals almost vertically, head down, and thrusting the needle into the abdomen in the median line a little above the bladder. The needle should not be too sharp, nor thrust in very deeply. (Personal communication of Dr. R. Krause.) The repetition of *intravenous* injections offer especial difficulties, for after hæmolysin formation has once occurred the blood-cells introduced are rapidly dissolved, leading to the death of the animal from embolism. (Rehns.<sup>1</sup>)

Another thing which may lead to death from embolism is the formation of coagulins in consequence of a previous injection of blood which has not been freed from serum. These coagulins cause a rapid formation of precipitates within the blood circulation.<sup>2</sup>

The amount of blood used depends upon the size of the animal to be injected and upon the special conditions of the experiment. Up to a liter of blood, freed from most of its serum, can be injected

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<sup>1</sup> Rehns, Comp. rend. de la Soc. de Biol. 1901, No. 12; see also similar observations made on man by Bier, Münch. med. Wochens. 1901, No. 15.

<sup>2</sup> Very likely the inexplicable results obtained by Magendie ("Vorlesungen über das Blut," German translation by Krüpp, Leipzig, 1839) were due to the formation of coagulins. Magendie found that rabbits which had tolerated two intravenous injections of egg albumin without any injury whatever immediately succumbed to a further injection made after a number of days.

into goats without injury. In rabbits of 2 kilos it will hardly be possible to go beyond 100 cc.; and guinea-pigs, corresponding to their weight, proportionately less. According to our experience a single injection of 20-30 cc. sheep, goat, ox, or dog blood leads to a strong formation of hemolysin, which can be still further increased by a subsequent injection of 40-60 cc. six to ten days later. We have found that further injections of the same or larger amounts (80-100 cc.) have no advantage. We have occasionally observed that these were associated with a *decrease* in the amount of hemolysin. As a rule, the serum attains its maximum power between the sixth and tenth day,<sup>1</sup> but this is subject to individual variations, as is shown by the case described by Ehrlich and Morgenroth of a goat in which an *ecolysin* developed critically on the fifteenth day (see page 29).

The injections of serum lead principally to the production of *antiamboceptors* and *anticomplements*, in some instances also to that of hemolytic *amboceptors* in consequence of the receptors present in solution in the serum.<sup>2</sup> The production of antiamboceptors necessitates a special selection of the animal species. Our own positive results are limited to the injection of goats either with the serum of a rabbit which had been immunized with ox blood, or with an *ecolytic* serum. Since in these cases the immune serum is toxic for the goat, or, more particularly, acts destructively on the blood, it is necessary to commence with the injection of small amounts (10-20 cc.) and gradually, as the reaction subsides, go on to larger doses. As in the case of all immunizations with toxic substances it is particularly necessary to keep a careful control of the weight of the animals; the rule always to be observed is that immunization can only then be proceeded with when the animal has again attained the weight it originally possessed.

In order to produce *anticomplements* larger animals, such as sheep and goats, are injected with increasing amounts of normal serum, beginning usually with fairly large amounts—100 to 500 cc. As a rule, when rabbits have been injected two or three times with guinea-pig, horse, goat or ox serum (commencing with 5 to 10 cc. and increasing to 20 to 50 cc.), a plentiful supply of anticomplement will have developed in the serum. In many cases the injection of an inactive

<sup>1</sup> See also Bulloch, *Centralblatt f. Bact.*, Vol. 29, 1901.

<sup>2</sup> See Morgenroth (page 241, this volume) and P. Müller, *Munch. med. Wochenschr.*, 1902, No. 32.

serum, which had thus been deprived of much of its toxic property, would appear to be preferable, for, owing to the complementoids which it contains, this would cause the production of anticomplements just as well as fresh serum. (See pages 79 et seq.)

If it is desired by the injection of a certain serum to produce anticomplements which are also directed against various other sera,<sup>1</sup> it is necessary to repeat the injections several times in increasing amounts. While treating a goat with rabbit serum, Ehrlich and Morgenroth observed the development first of anticomplements directed exclusively against the complement of rabbit serum (isogenic anticomplements); in course of time anticomplements directed against the complements of guinea-pig serum (alloiogenic anticomplements) also appeared. Here evidently we are dealing with partial complements, present in rabbit serum in small amounts, which require several repetitions of the injections in increasing amounts in order to excite the production of anticomplements.

In the production of serum coagulins [precipitins] one proceeds as for anticomplements. These serum coagulins have been shown to possess considerable value for the forensic determination of various species of blood, especially human blood, as has been shown by the researches of Wassermann and Schütze, Uhlenhuth, and many others. In the production of milk coagulins one or two injections of 20 to 40 cc. of milk into a rabbit are usually sufficient. The milk can be heated to 60° previous to injection in order to reduce the number of germs present. In connection with the production of serum coagulins Uhlenhuth makes some interesting statements (*Deutsch. med. Wochenschr.* 1902, No. 37). Among other things he describes something we had also noticed, namely, the occasional failure of the reaction and the development of "alloiogenic" coagulins as the titer of the serum increased, a fact which corresponds to what we have above described for the formation anticomplements.<sup>2</sup>

#### IV. Determining the Hæmolytic Action.

The fact that certain poisons of vegetable or animal origin, as well as normal sera and other body fluids, possess a hæmolytic action can be determined so readily that it will be superfluous to enter further

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<sup>1</sup> See pages 111 et seq.

<sup>2</sup> Concerning isogenic and alloiogenic anticomplements, see Morgenroth and Sachs, pages 258 et seq.



into the subject. In passing, however, it may be mentioned that for an investigation in this direction to be at all complete it is necessary to make use of as many different species of blood-cells as possible. The susceptibility of the cells can be extraordinarily diverse, so that certain poisons exert a marked hemolytic effect on some species of blood, while they fail to have any action whatever on other species. Thus the poison of the garden spider, studied by Sachs,<sup>1</sup> is inert for guinea-pig or dog blood cells, while it has strong hemolytic powers for rabbit blood cells. Crotin which dissolves certain blood-cells (e.g. rabbit blood) and agglutinates others (e.g. hog blood) behaves in similar fashion.<sup>2</sup>

In the case of the specific hemolysins produced by immunization the choice of blood, of course, is already indicated. But even here, extending the investigations to numerous other species of blood may lead to valuable information concerning a community of receptors such as exists between sheep, goat, and ox,<sup>3</sup> and as has recently been shown by Marshall to exist between man and certain species of monkeys. In testing a serum for the presence of hemolysins it is necessary to use the blood of numerous individuals, for according to our experience the sensitiveness of the blood, in the case of goats, is subject to the widest individual fluctuations. In this way one can easily be misled to assume that the experiment results negatively. It is advisable, when testing a fluid for hemolytic properties for the first time, to remove the serum by washing the blood-cells at least once. Under certain circumstances a slight degree of hemolytic action can be masked by an antihemolytic action of the normal serum. This is seen to a high degree in the case of the hemolytic poisons of the organ extracts.<sup>4</sup> So far as the dosage is concerned one should select wide limits, especially in the first experiments. If one has once determined the presence of a hemolytic action, the quantitative estimation follows by means of a more or less finely graded series of experiments. Types of these experiments are found on pages 168, 270, 276, etc.

In testing a hemolysin which has not yet been examined, it is

<sup>1</sup> See pages 167 et seq.

<sup>2</sup> Elfstrand, *Über giftige Eiweissstoffe welche Blutkörperchen verkleben*. Upsala, 1891.

<sup>3</sup> See pages 93 et seq.

<sup>4</sup> See Korschun and Morgenroth, pp. 267 et seq.

important to determine whether the hæmolytic agent is a haptin in the true sense. So far as the alkaloids, glucosides, etc., which act hæmolytically are concerned, they are generally readily identified by means of the chemical methods devised for their separation, methods based on precipitations and shaking out with solvents. This is not true for the haptins; they cannot be prepared by these methods. At the most, it is possible to precipitate them in conjunction with the albuminous bodies. Another distinction consists in this, that the substances which are chemically defined are usually thermostable, while the haptins in the great majority of cases are destroyed by heat, especially by boiling temperature. One distinction above all, however, is the fact that only the haptins are capable of causing the production of antibodies by immunization, and this makes a classification possible even in difficult cases. Frequently the facts which we have already learned about a substance allow us to make definite conjectures. For example, if a vegetable extract possesses hæmolytic properties which are not destroyed by boiling, and if it is found that the hæmolytic substance is soluble in ether, we can at once exclude this from the class of haptins. On the other hand, if one finds that the hæmolytic action of an animal body fluid is destroyed by heating to 56° C., this fact already argues in favor of a haptin. Other methods, including perhaps the immunizing reaction, would then be required to determine this positively.

#### V. The Study of Complex Hæmolysins.

We now take up a question of paramount importance which arises in the study of every hæmolytic poison, namely, whether in any given instance we are dealing with a simple hæmolysin, or with a complex one consisting of amboceptor and complement.

In determining the complex nature of a hæmolysin we now have the following methods at our disposal:

1. Separation of amboceptor and complement by allowing the former to be tied by red blood-cells at low temperatures.
2. Removal of the complement or changing the same into the inert complementoid.

(a) Absorbing the complement by means of certain cells (e.g., yeast-cells, bacterial cells, cells of animal organs), or by means of porous filters.

(b) Thermic and chemic influences, such as heating to 50-60° C., the action of alkalis and acids, digestion with papayotin.

The separation of antibody and complement at low temperatures is of the utmost importance and has been used for the analysis of complex hemolysins with considerable success. The conditions necessary for the successful operation of this method have been discussed in detail in a previous paper. A separation is only then possible when at low temperatures the affinity between the cytoplasmic group of the antibody and the receptor is greater than that between the complementoplasmic group of the antibody and the corresponding group of the complement. The degree of difference in the affinities would, of course, determine the degree of completeness of the separation. In some instances most peculiar relations are found, as is shown, for example, by the behavior of red serum to rabbit blood. Attempts to effect separation at low temperatures fail in this case, first, because hemolysis ensues even at 0° C., and second, because the employment of higher concentrations of salt (up to 5%) which in other cases has afforded a means of loosening the combination of antibody and complement, does not suffice to prevent hemolysis. Naturally from this behavior we must not conclude that red serum does not contain a complex hemolysin, but merely that in this case peculiar conditions are present which, owing to the insufficiency of the methods thus far employed, are still obscure to us. In those cases in which separation at low temperatures fails, a second method may be considered. This depends on the fact that a high degree of salt concentration, somewhat after the manner of low temperatures, can prevent hemolysis; concentrations which still permit the union of receptor and antibody preventing that of antibody and complement. The prevention of hemolysis by means of salts, first described by Markl<sup>1</sup> and erroneously ascribed by him to conditions of diffusion, is also due to this. Markl entirely overlooked the fact that in certain cases the combination of toxin and antitoxin (e.g. Tetanus toxin + Antitoxin) is also prevented by salt (Knorr). For the application of this method see Ehrlich and Sachs, page 214.

It is perfectly obvious that the cold method will fail absolutely in cases like the one described by Ehrlich and Sachs (page 217) in which the union of antibody and complement is the prerequisite

<sup>1</sup> Markl, *Zeitschr. für Hygiene*, Vol. 39, 1902.

for the union with the blood-cells. Such a possibility must be borne in mind.

The technique of this separation at low temperatures is simple. The tubes containing the blood and the serum respectively are cooled to  $0^{\circ}$  by being placed in iced water or by packing in ice. Thereupon the serum, in amounts which are not far either way from the single solvent dose, is added to the blood. After being kept at  $0^{\circ}$  for two hours the mixture is rapidly centrifuged and the supernatant fluid quickly removed. If desired, the sedimented blood-cells can be washed with salt solution and then suitably suspended. The decanted fluid is again mixed with blood-cells. For this purpose, in order not to increase the total volume, one takes the blood-cell sediment centrifuged from the required amount of the suspension. If a complete separation of amboceptor and complement has been effected, it will be found that neither are the sedimented blood-cells dissolved nor is the decanted fluid able to dissolve blood-cells added anew. It is then necessary to determine the presence of complement in the decanted fluid, which is done by adding suitable amounts of serum inactivated by heating. Similarly amboceptor anchored by the blood-cells at low temperatures is demonstrated by adding to the sediment the complement present in the decanted fluid.

The second and simpler method is that of inactivating the hemolytic serum by means of heat and then activating the amboceptor by the addition of complement. In this the chief difficulty consists in the fact that a certain complement required in a particular instance is not contained in all sera, and further that the sera which contain this particular complement often in themselves dissolve the blood-cells by means of a normal amboceptor.

There are several ways of overcoming these difficulties. The nearest method and one which is applicable in many cases consists in selecting as the complementing agent the serum of that species whose blood is being tested, as, for example, using guinea-pig serum as complement for amboceptors acting on guinea-pig blood. In such cases a solution of the blood-cells by means of the animal's own serum is, of course, precluded.

In all other cases one must make use of complementing sera which are unrelated to the species of blood in question. One frequently discovers sera for this purpose which do not in themselves dissolve the blood-cells to be tested, as, for example, in reactive

the antioptors for sheep blood or ox blood by means of goat serum.

But it is often possible to complement an antioptor with a serum which in itself dissolves the blood cells, but which, in the amounts in which it is able to effect completion, has little or no hemolytic action. It is obvious that in such cases the solvent power of the serum by itself must be accurately determined by means of controls. While this method is often successful, the relation in these sera between the normal antioptor and the complement is frequently so unfavorable that it is impossible to complement the foreign antioptor. In such cases one can get rid of the normal antioptor by allowing it to blood cells at low temperatures, as Fleener and Noguchi<sup>1</sup> have recently done in order to obtain complements for the hemolytic antioptors of snake venoms. Or one can attempt artificially to increase the amount of complement contained in complementing serum, after the method of P. Møller.<sup>2</sup> This author succeeded in effecting a considerable increase in the complements of chicken serum, by injecting the animals with solutions of peptone.

So far as the choice of the complementing sera is concerned it is obvious that, in antioptors produced by immunization, whenever possible the preference will be given to those sera which are derived from the same species which yielded the antioptor. For the remaining cases the principle may be formulated that that serum is most useful which is derived from a species closely related to that furnishing the antioptor, because often in distantly related species partial antioptors present only in very slight amounts are complemented.<sup>3</sup>

Another point of considerable importance in the completion of antioptors is the manner in which the sera are inactivated. As a rule inactivation is effected by heating the serum for half an hour in a water-bath. According to recent investigations special attention must be paid to the degree and duration of this action.<sup>4</sup>

<sup>1</sup> Fleener and Noguchi, *Journal of Exp. Medicine*, Vol. VI, 1902.

<sup>2</sup> Møller, *Centralblatt f. Bacteriologie, Orig.* Vol. 29, 1901.

<sup>3</sup> Ehrlich and Morgenroth, see pages 110 et seq.

<sup>4</sup> In order accurately to observe the temperature constantly maintained it is well to use thermometers with particularly wide divisions on the scale ( $1^{\circ}\text{C} = 1\text{ cm}$ ). These thermometers need only embrace a moderate range of degrees (about  $40^{\circ}$ - $50^{\circ}$  or  $45^{\circ}$ - $55^{\circ}$ ). They can be obtained from A. Haak in Jena.

For many years, owing to the valuable researches of Buchner, an inactivation by means of temperature of 55-56° was regarded as practically a specific criterion for the alexins. We now know, however, that no general rule can be formulated in this respect. On the one hand there are complements which are not at all influenced by the customary half-hour's heating to 55° C. (thermostable complements), and on the other there are amboceptors which are completely destroyed by such heating. A complement belonging to the first category was first described by Ehrlich and Morgenroth,<sup>1</sup> as occurring in considerable amount in normal goat serum and in the serum of a buck which had been immunized with sheep serum; and thermolabile amboceptors, especially in normal sera, are not at all rare. Thus the amboceptor above mentioned regularly present in horse serum and acting on guinea-pig blood, as well as one studied by Sachs<sup>2</sup> present in dog serum and also acting on guinea-pig blood, is completely destroyed by half an hour's heating to 55° C. Hence the first rule in the demonstration of the complex character of hemolytic poisons by thermogenic inactivation is always to employ the lowest temperature at which inactivation takes place within a short time (20-60 minutes).<sup>3</sup>

#### VI. The Quantitative Estimation of Amboceptors, Complements and Receptors.

In special cases, e.g. during the course of an immunization, it is of considerable value to accurately determine the amounts of amboceptor and complement present in the serum. While referring to the studies of v. Dungern (p. 36), Bulloch (l.c.), Morgenroth and Sachs (pp. 226 and 250), we should like to emphasize that, in general, in determining the amount of complement it is necessary to make

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<sup>1</sup> See page 13.

<sup>2</sup> See page 181 et seq.

<sup>3</sup> According to the researches of Korachun and Morgenroth (see pp. 267 et seq.) the hemolytic substances of organ extracts are "coctostable," i.e., they are not destroyed even by several hours' boiling. Hence we designate a substance as

*Thermolabile*, if it is rendered inert by heating to 55°-56° C.;

*Thermostable*, if it withstands heating to 56° or over but is destroyed by boiling;

*Coctostable*, if it resists boiling at 100° C.

In special cases in order to still more closely characterize their behavior one can add temperature and duration of heat as an index.

two determinations, namely, one carried out with the single-solvent dose of ambocceptor, the other with a high multiple of the same. The reasons for this procedure can be found in the study on the quantitative estimation of ambocceptor, complement, and anticomplement (page 250).

So far as the estimation of the amount of ambocceptor is concerned, this is effected according to similar principles, and usually in such a way that one works with an excess of complement. A certain difficulty is encountered in the fact that the amount of complement contained in the serum, e.g., rabbit serum, is variable. It is therefore always necessary, in order to exclude this disturbing factor, to first determine the activating value of the complementing serum using a specimen of the immune serum in question as a standard serum. Directly after this test by which the amount of complement is strictly defined, the quantitative estimation of ambocceptor in the new serum must be undertaken.

It is also important to estimate the amount of receptor present in the red blood-cells. The measure of this is the binding of ambocceptor.

Ehrlich and Morgenroth (see pages 72 et seq.) have demonstrated that the binding capacity of red blood-cells varies to an extraordinary degree. While in many combinations the blood-cells combine with just that amount of ambocceptor, which on the addition of suitable complement leads to their complete solution (*ambocceptor unit*), it was found that in numerous other cases the blood-cells are able to take up as high as 100 single-solvent doses of ambocceptor. Corresponding to the ambocceptor unit, the *receptor unit*, is that amount of receptor which combines with one ambocceptor unit (see page 254). The combining power of the erythrocytes is determined by adding varying multiples of the ambocceptor unit to the blood-cells, centrifuging at the end of about an hour and then allowing the various decanted fluids to act on fresh blood-cells in the presence of sufficient complement. The degree of hemolysis which occurs readily shows just how much ambocceptor was still completely bound. (See page 75 and the protocols on pages 98 and 99.)<sup>1</sup>

<sup>1</sup> Concerning the extraordinarily large binding capacity of bacteria for agglutinins and for ambocceptors, see the interesting communication of Eisenberg and Volk (Zeitsch. f. Hygiene, Vol. 80) and of Pfeiffer and Friedberger (Berl. klin. Wochensh. 1902, No. 25).



Finally, in studying the complements of a serum it is often of considerable importance to determine their plurality. The methods leading to a differentiation of the separate complements have been described in detail in a number of places, so that we can here content ourselves by referring to the studies of Ehrlich and Morgenroth (pages 11-56, 110), of Ehrlich and Sachs (page 195), and of Marshall and Morgenroth (page 222).

### VII. The Study of Antihæmolytic Actions.

The subject of antihæmolytic functions, which has only recently been carefully worked up, has attained considerable importance for the comprehension of the mechanism of hæmolysins. Although at the present time the study of the influences inhibiting hæmolysis is not at all complete, it is possible at least to indicate certain general principles.

We shall begin with the simple hæmotoxins, which are characterized by a cytophile haptophore group and a zymotoxic group. (Analogous to these are the hæmagglutinins, also characterized by a cytophile haptophore group and an agglutinating group.) If we analyze the action of these hæmotoxins, we see that this can be inhibited in two ways:

(1) By means of an antibody which fits into the haptophore group and so deflects this from the receptor of the cell.

(2) By means of substances which are capable of occupying the receptor of the blood-cell and so block this for the entrance of the hæmotoxin.

So far as the first group is concerned, such antibodies are well known for a large number of blood poisons. We need only call to mind the antihæmolysins, such as anticrotin, antitetanolysin, antistaphylolysin, antibodies against the hæmolytic venoms of snakes, spiders, and toads. Besides these there are the antiagglutinins, such as antiricin, antiabrin, anticrotin. These substances can be produced as antitoxins by means of immunization, but they also occur in normal serum, as, for example, antitetanolysin in horse serum (Ehrlich), antistaphylolysin in serum from goats, man, and horse (M. Neisser and Wechsberg).

The second method of inhibition is effected by substances which occupy the receptors of the cells. Hence these must be substances which possess the same haptophore group as the hæmotoxins them-



subject. This, however, at once leads to the idea that transformation products of the hemolysin itself could exert this action. Ehrlich's researches on the constitution of diphtheria poison have shown that in toxins and related bodies the asymptotic group is far less stable than the haptophore group. The bodies so derived, *toxoids*, still possess the property of combining with the cell receptors, they are still able to neutralize antitoxin, and to excite the reactive formation of antibodies, but they more or less completely lack any toxicity. This formation of *toxoid*, first described by Ehrlich, has since been demonstrated for a number of substances, hemotoxins (tetanolyxin, snake venom, staphylotoxins), as well as agglutinins and coagulins.<sup>1</sup> Ehrlich in his first study already pointed out that an increase in the haptophore's affinity, developing in the course of *toxoid* formation, was conceivable. The *toxoid* which was thus produced would then be able, owing to the increased affinity, to unite with the receptor of the cell even in competition with the unchanged toxin. In this way the *toxoid* would protect the cell against the entrance of the real poison, and of course, against the poison's injurious influence. For these *toxoids* Ehrlich has proposed the term *pro-toxoids*. Of course such a protective effect can also be produced in conformity with the laws of mass action by *toxoids* having the same affinity (*syntoxoids*) to the cell receptor as the toxin, whereas the protection will be slight or minimal if, as a result of *toxoid* formation, there is a decrease in the haptophore group's affinity (*epi-toxoids*). Recent investigations on the agglutinins of bacteria<sup>2</sup> and on coagulins have shown that by heating these substances, agglutinoids, which possess a higher affinity than the agglutinins themselves, are developed in considerable quantities. These are, therefore, termed *proagglutinoids*.

It is an easy matter in any given instance to determine experimentally which of these two inhibitory processes is present. If one is dealing with a certain particular serum which inhibits the action of the hemotoxin, it may be regarded *a priori* as probable that the substance in question is an antibody in the ordinary sense. This becomes almost certain if the serum was derived from an animal specifically immunized. Experimentally it is easy to show that

<sup>1</sup> Eisenberg and Volk, *Zeitsch. f. Hygiene*, Vol. 40, 1902; Ball, *Archiv f. Hygiene*, Vol. 42, 1902; Shiga, page 312.

<sup>2</sup> *Ibid.*

the antibody belongs to this group as follows: The red blood-cells are treated with a just neutral mixture of hæmotoxin and antibody and then centrifuged. If there was a true deflection of the poison, these cells must now behave exactly like fresh blood-cells; above all they must still possess exactly the original binding capacity for the hæmotoxin.

In contrast to this behavior, the disturbance caused by transformation products of the hæmolysin itself manifests itself even in experiments made only with blood-cells and the toxic substance. The experimental series has an irregular course analogous to Shiga's experiments with agglutinins. For example, if increasing amounts of agglutinating serum which has *previously been heated* are added to dysentery bacilli, one can observe that the test-tubes containing the largest amount of agglutinins show no agglutination; and that agglutination shows itself only in the tubes containing smaller amounts and disappears again with still smaller quantities.





In order to show that in this case there is no real occupation of the receptors by the proagglutinoid, one tests the behavior of the centrifuged bacteria. These are suspended in salt solution, and again mixed with what is otherwise an effective dose of agglutinin. They are no longer agglutinated because the agglutinin cannot combine with the blocked receptors. We do not doubt at all that this phenomenon will also be found in hæmagglutinins.

The conditions are far more complicated with the complex hæmolysins, the possibilities for the inhibitory mechanism being more numerous. It may, therefore, be well to aid our analysis by means of a diagram (see opposite).

The diagram refers to experiments made with mixtures which do not by themselves dissolve blood-cells, and whose composition must first be accurately determined quantitatively. One next devises a hæmolytic combination in which amboceptor and complement are present in exact equivalence and determines the amount of the antibody in question which will just inhibit the action of this combination. By means of this exactly balanced mixture experiments by the centrifuge method are made both with the sediments and with the decanted portions as shown in the diagram.<sup>1</sup>

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<sup>1</sup> This method refers to cases I, III, and IV of the scheme, while case II refers to an experiment made with ordinary complementoid serum obtained by heating.

		Behavior of the Sediment.		Behavior of the Decanted Fluid		
		Agglutinating Power by Means of New Sample, 5 sec.	Agglutinating Power by Means of Amblycyte, 10 min.	Amblycyte Coagulation, 10 min.	Coagulation of Complement, 10 min.	Coagulation of Complement, 10 min. (same as 5 sec.)
 <p>I.</p> <p>Anticomplement c, complement ac, anticomplement</p>		+	0	0	0	
 <p>II.</p> <p>Blocking of the complementophilic group of the amboceptor (ac) by means of complement (c)</p>	at 0°	+	0	0	0	+
	at 37°	0	0	0	0	0
 <p>III.</p> <p>antiamboceptor (ac) a, amboceptor</p>		0	+	0	+	
 <p>IV.</p> <p>cytophilic protomamboceptor (ca), receptor of the red blood-cell, c.</p>		0	0	+	+	

In studying the sediments the question is always whether these have taken up amboceptor or not. This is most easily determined by the addition of complement. This procedure, however, should be supplemented by the more difficult and troublesome investigation of the binding power of the blood-cells for newly added amboceptor. In this case, of course, a parallel test with untreated blood cells furnishes the basis for comparison. As a rule experiments at moderate temperatures suffice; only in case II is a variation of temperature required.

In case I the complement is deflected by means of an anticomplement. One must take into consideration both natural anticomplements and those artificially produced by immunization; furthermore, attention must be paid to similarly acting derivatives of the amboceptors, the amboceptoids, whose complementophile group has been preserved.<sup>1</sup> The behavior of the amboceptoids, especially in those cases in which the affinity of the complementophile group of these amboceptoids has become increased, will in no way differ from that of the anticomplements. Finally we must remember that the amboceptors can act in a way like anticomplements as a result of the deflection of complements by excess of amboceptor, a phenomenon first described by Neisser and Wechsberg (see page 120). In that case, of course, the decanted fluid contains the excess of amboceptors and the complement bound to the same.

II. In case II the complementophile group of the amboceptor is blocked. Here we must first consider the action of complementoids (see Ehrlich and Sachs, page 200), although, according to our present experiences, these only seldom come into play because, in the formation of complementoid, there is usually a *decrease* of affinity.

III. The third possibility is the action of antiamboceptors which fit into the cytophile group of the amboceptors. They may be present normally or produced artificially by immunization. From a theoretical standpoint these antiamboceptors are to be identified with the receptors of the cells into which the amboceptors fit. Hence thrust-off receptors present in solution will act as antiamboceptors.<sup>2</sup> According to recent investigations the serum against snake venom

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<sup>1</sup> Wechsberg, Wiener klin. Wochenschr. 1902, No. 28; E. Neisser and Friedemann, Berl. klin. Wochenschr. 1902, No. 29.

<sup>2</sup> Morgenroth, page 241; also P. Müller, Munch. med. Wochenschr. 1902, No. 32.

also contains antianibocceptors against the anibocceptors of cobra venom.

IV. The fourth possibility consists in the occupation of the receptor by cytoplasmic *proanibocceptors*, conditions which correspond to those discussed under the simple hemotoxins (page 342). Since the study of anibocceptors is still in its infancy, such cases have not yet been described. Their occurrence, however, is extremely probable and the near future will probably furnish experiences in this direction.

So far as the details of the experiments are concerned, the previous papers furnish detailed descriptions which may be consulted. The reader is referred to the following: Case I, pages 224-259; II, page 269; III, pages 103, 104, 248.

In any particular instance it is necessary to determine which of these cases obtains. Above all it is necessary to remember that the inhibition need not always be due to a specific binding, but that it may be caused by disturbing factors, which we have classed together under the term *antireactive actions*.

For example, if the union of anibocceptor and complement does not take place at low temperatures, or if owing to the action of salt the union of complement with the anchored anibocceptor, or of anibocceptor to the cell receptor, is hindered, these are the result of *antireactive influences* and not of specific inhibitions. As a rule it is easy in any given case to decide which kind of inhibition is present. In most cases the origin and mode of derivation of the substances in question give valuable clues in this direction. If antireactive influences can be excluded, it is not difficult by a logical application of the centrifugal method to classify the cases under one of the heads given in the table.

Naturally these cases may also be combined. Thus, for example, a fluid may contain simultaneously anticomplement, anti-anibocceptor or anticomplement and proanibocceptor. Anti-anibocceptor and anibocceptor, complement and anticomplement in *one* solution can be excluded, since they mutually neutralize each other.

Another important point which belongs here is the recognition of concealed anibocceptors, whose activatability is suppressed by the simultaneous presence of anticomplement. For the experimental technique see Morgenroth, page 245.

It is, of course, impossible to treat exhaustively all the innumerable variations which come into question. We hope, however, that the methodical exposition here given has shown how the fundamental doctrines of Ehrlich's Side-chain Theory make a systematic study of hemolysis possible.

### XXX. THE TECHNIQUE OF BACTERICIDAL TEST-TUBE EXPERIMENTS.

By Professor M. NEISSER, Member of the Institute.

IN order to measure the bactericidal power of a serum or of serum mixture by means of a test-tube experiment, the plate method (Neisser, Buchner) is still the safest. Only in special cases can one obtain useful comparative results by other methods (observing hanging drops for the onset of granular degeneration, R. Pfeiffer, or bioscopic method, M. Neisser and Wechsberg<sup>1</sup>). But even the plate method at present is cumbersome and, what is of more consequence, is not applicable in all cases. It is not a sensitive method and is only then useful when marked results are to be expected in consequence of strong bactericidal powers. As a rule such marked results are only to be attained with immune sera and only rarely with normal sera.

So far as *immunization* is concerned it is impossible to make general statements, and I shall therefore only cite a few examples. Thus in the case of cholera vibrios a single subcutaneous injection of three dead agar cultures into rabbits gives good results (R. Pfeiffer and Marx<sup>2</sup>), as does also the intravenous injection of extremely small quantities (Mertens, R. Pfeiffer<sup>3</sup>). In immunizing against typhoid, dogs and goats are most useful. In this case a single injection of dead cultures does not suffice in order to obtain a high-grade bactericidal serum; on the contrary repeated injections of living cultures are necessary. For obtaining a serum having strong bactericidal properties against Shiga's dysentery bacilli, horses are well adapted; goats very much less so; rabbits and guinea-pigs are very

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<sup>1</sup> Münch. med. Wochensh. 1900, No. 37.

<sup>2</sup> Zeitschr. f. Hygiene, XXVII, 1898.

<sup>3</sup> Deutsche med. Wochensh. 1901.

ill-suited for this purpose. One should, of course, never forget to examine the normal serum for bactericidal powers previous to immunization.

With a great many bacteria it has not yet been possible to produce a serum bactericidal *in vitro*. Thus our experiments in this direction extending over many years were unsuccessful with *staphylococcus pyogenes aureus* (goat, rabbit) and with the diphtheria bacillus. Nor have we been able thus far to obtain bactericidal effects *in vitro* from Susserin and other similar sera which are effective in animal tests. The reasons for this behavior are not yet clear, and they are therefore still being studied.

Bordet and Gengou have devised a method (Annales de l'Institut Pasteur 1901) by the aid of which a bactericidal interbody produced by immunization can be recognized even in those cases in which plate experiments fail (e.g. erysipelas of swine). This method depends on the property, said to be possessed by bacteria to which interbody has been supplied, of combining also with hæmolytic complements. This loss of complement, which can be readily detected, shows that the bacteria have combined with a bactericidal interbody. Without entering into the theoretical significance of this interesting experiment we shall content ourselves by saying that in several cases in which we tested bactericidal immune sera in this way we failed to obtain satisfactory results. The method does not seem to us to be suited to a quantitative estimation of an immune serum.

It need hardly be said that the first requisite for the success of bactericidal experiments is that all vessels, diluting fluids, as well as the sera employed be absolutely sterile. Great care is necessary, especially in collecting the blood. The method described in the preceding chapter for bleeding rabbits and guinea-pigs is sufficient to obtain sterile blood. For collecting smaller quantities of blood from the ear vein of rabbits it is necessary to first cleanse the ear with 70% alcohol and then thrusting a short sterile hollow needle into a vein. In many cases, to be sure, the blood can also be collected by making a short incision across the marginal ear vein with a sterile scalpel, and then, by holding the animal properly, allowing the blood to flow out without running over the ear.

In bleeding pigeons and chickens by decapitation one cannot always count on sterile serum; hence it is well to lay bare the vessels of the neck. For repeated bleeding of guinea-pigs one must also



collect the blood directly from the vessels of the neck, and then tie the vessel. It is an easy matter to obtain very small quantities of sterile pigeon blood from the wing veins by first carefully removing the feathers, disinfecting the skin with alcohol and then after incising, touching the skin as little as possible.

For purposes of collecting the serum, the blood is either allowed to stand overnight (see the preceding chapter), or by means of a sterile funnel is allowed to flow into a sterile bottle containing sterile glass beads or steel shavings. The bottle is then stoppered with a cork (previously burnt off), the blood defibrinated by shaking, and then centrifuged. As a rule, centrifuging does not injure the serum, especially if afterwards the upper layer of fluid is siphoned off. For absolutely certain sterility the spontaneous separation of the serum is to be preferred to defibrination and centrifuging.

The active sera used for complementing are to be employed as fresh as possible, in no case more than two or three days old (refrigerator). The immune sera, which are usually employed in the inactive state, will keep in the refrigerator for a long time. Even in these, however, a loss of power is observed. In the case of high grade immune sera the addition of 0.5% phenol is allowable for preservation. In the small quantities in which the serum is used in experiments (about 0.01 cc.) this amount of phenol is without effect either on the bacteria or on the complements.

Before commencing the experiment proper it is necessary to determine what amount sown gives the most favorable results. Thus in many experiments it may be of advantage to always sow  $\frac{1}{500}$  cc. of a one-day bouillon culture, whereas with another bacterium sowing  $\frac{1}{1000}$  or  $\frac{1}{10000}$  loop of a one-day agar culture will give more uniform results. It is further necessary to repeatedly convince one's self that the control plates regularly show a uniformly good growth, for only when that is the case can uniform results be expected. For example, although the bacillus of hog cholera grows very well on ordinary slant agar, the control plates may result most irregularly. In that case one can make use of glycerine agar. Other bacteria again do not bear suspension in 0.85% salt solution at all well; in that case one must use bouillon cultures and make the dilutions with bouillon instead of with salt solution. The dilution should always be managed so that the amount finally sown is about 5-10 drops, for in sowing only 1 or 2 drops considerable variations in the number of colonies may occur. In any case, however, the



plate shown must contain many *thousands* or an *innumerable* number of colonies. The bactericidal effect will then be distinctly shown by the reduction in the proper plates of this large number of colonies to zero or almost zero.

The test tubes most advantageously employed are the little tubes 9.10 cm. long and 1.5 cm. diameter. The cotton stoppers are removed and all the different components filled into the tubes. Then the stoppers are replaced after being flamed. If the air is at all still one need not fear keeping the tubes open for this length of time.

In testing an immune serum one commences by examining the immune serum in the fresh active state, and, of course, in the same manner that the serum of the animal in question was examined previously to immunization. For this purpose a number of test tubes are filled with 1.0, 0.4, 0.1, 0.02, 0.01 cc. of the fresh active serum. Inner graduations are useless in view of the lack of sensitiveness of the test-tube method. This we have already pointed out. The amount of culture to be sown is then added and all tubes filled up to 2 cc. with physiological salt solution. Finally three drops of bacillon are added to each tube. The addition of bacillon has proven to be of considerable value, for it suffices to balance disturbing variations of the osmotic pressure. It is important to make the total volume of fluid the same in all the tubes by the addition of fluid. Besides this it is important to have a number of controls, namely, a control of the culture sown, second, a control testing the sterility of the maximum amount of serum employed, and third, a control, or better a series of controls, containing the culture sown plus the serum in an inactive form. By means of this last control one can see whether a thermostable complement is present or not. It also serves to show that the bactericidal action is not stimulated by the agglutinating power of the serum.

The tubes are now kept in the thermostat for at least three hours, having previously, however, been carefully shaken. On being taken out of the thermostat they are again carefully shaken and then worked up into plates. For this purpose 5-10 drops are taken from each tube by means of uniform pipettes and made into plates in the usual way. The plates are placed in the thermostat upside down, and kept there until the following day. The growth is best and most rapidly described by means of approximate estimates, using a scheme somewhat as follows: 0 or almost 0, about 100, several hundreds, thousands, very many thousands, infinite number. A distinct bactericidal action is only then present if the controls result as they

should, and if a reduction of colonies from an infinite number or many thousands to 0 or very few has occurred. Furthermore the test can only then be regarded as having a good result if the lower limits of the amount of active serum have been reached, i.e., when the last plates again show an increase in the number of colonies.

A certain degree of control on the plate experiments is obtained in suitable cases by placing the tubes (from which a few drops were taken for sowing into plates) into a thermostat and observing them the next day. In this case the culture controls show a luxuriant growth, while in the other test-tubes, depending on the amount of serum, either a growth will occur or not. This test-tube experiment, of course, will only then show a result if the bactericidal power of the serum was large enough to kill even the last germ in the corresponding specimens. But if even only a few germs remain alive (in consequence, for example, of a special resistance), it will be found that these few, after the bactericidal substances are used up, will again multiply enormously. Hence the test-tube method cannot give reliable results in spore-bearing bacteria. For the same reason it is important, in making plate tests, to keep the tubes in the thermostat for a certain particular time, which must be determined separately for each bacterium; for it must be borne in mind that the killing of the bacteria can be represented by a curve whose lowest point (lowest number of living germs) must be approximately attained if marked results are desired. Either side of this point, unless this point be 0, the results will be correspondingly less. Smaller results, however, are worthless for all these experiments, as is seen when we consider that agglutination, although it has so little directly to do with bactericidal action, is also able to cause a decrease in the number of colonies on a plate and thus simulate a decrease in the number of germs. This is one of the reasons why the control described above with inactivated serum, in which, of course, the agglutinin is still present, is so important.

After the fresh active immune serum has been tested as to its bactericidal power one proceeds with the examination of the inactive immune serum plus complement. Inactivation is accomplished in accordance with the principles laid down in the preceding chapter. For complement one chooses first the normal serum of the species from which the immune serum is derived. A preliminary trial will then be necessary to show what dose of this normal serum can be

employed without causing bactericidal action by the normal serum itself.

The dose of complement should be such that the plate containing only complement and the culture differs very little from the control of the culture sowing alone. Too large a quantity of complement should be avoided; certainly in no case should more than about 0.5 cc. complementing serum be used. The technique then is as follows: 1.0, 0.3, 0.1, 0.03, 0.01 cc. of inactive immune serum are placed into a series of test-tubes; to each of these is then added the same amount of the complementing active normal serum (e.g. 0.3 cc.) and the bacterial culture. All of the tubes are then made up to the same amount (2 to 3 cc.) with physiological salt solution, and finally each tube receives three drops of bouillon. The controls in this case must be still more numerous. The sterility of each serum must be demonstrated, as well as the fact that the inactive immune serum by itself and the active normal serum by itself are inert.

The result of such an experiment is usually startling at first sight because the plates which had the largest amounts of immune serum show the largest number of colonies. One must therefore always bear in mind the deflection of complements in consequence of an excess of immune body. The paradoxical results caused by this deflection of complement is seen not only in the plates but also in the test-tube experiment. The various ways in which the complement is deflected from its destination have already been discussed in a previous chapter. In bactericidal experiments the deflection caused by an excess of the amboceptors produced by immunization is especially important. In a mixture of bacteria, complements, and large amounts of amboceptor, the complement is bound not only by the amboceptors anchored to the bacteria but also in large measure by "free" amboceptors which are not anchored to bacteria. A portion of the anchored amboceptor therefore finds no complement at its disposal and is, therefore, unable to exert any bactericidal action. In this way there arises a relative lack of complement. This can occur especially if part of the amboceptors has become changed into an amboceptoid with increased affinity (Wechsberg,<sup>1</sup> E. Neisser and Friedemann<sup>2</sup>). In bactericidal experiments, however, the cooperation of the amboceptoids has not yet been proved.

The completion of amboceptors can be disturbed in another way.

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<sup>1</sup> Wiener klin. Wochensch. 1902.

<sup>2</sup> Berl. klin. Wochensch. 1902.

Thus complement-diverting groups pre-existing in *normal* serum of the species in question, and which have not, therefore, originated through immunization, may be present or may be set free by the inactivation (normal anticomplements, etc.). The question which arises, namely, whether one is dealing with a deflecting body of *normal* serum or with one produced by *immunization*, can, of course, be decided by the previous investigation of the normal serum of the animal in question, as well as by comparison with several other normal sera of the same species.

In all of these cases, however, the plates with the largest amounts of immune serum will show the least bactericidal action, i.e., the largest number of colonies. From this it follows that one can err in judging the bactericidal power of a serum if only larger amounts of immune serum are used for the bactericidal test (about 1.0, 0.3). Thus in the beginning we overlooked the high bactericidal power of a dysentery serum (Shiga), for this became manifest only after we employed doses of 0.025 immune serum and still less.

The deflection of complement just mentioned, by means of amoebocytes produced by immunization (or by amoebotoxins), permits of another method of testing by which also the serum can be shown to be a specific immune serum. For this purpose one uses an active normal serum bactericidal in itself or a mixture of inactive immune serum and a complement. By means of a preliminary test one determines the amount of serum or serum mixture which completely kills the amount of culture sown. To such a dose of serum or serum mixture (bactericidal in itself) decreasing amounts of inactive immune serum are added, when it will usually be found that the phenomenon of deflection of complement again appears. This manifests itself by the fact that the plates with the larger amounts of immune serum show a larger number of colonies, the number of these decreasing in proportion with the amount of immune serum added.

In order to interpret the results of the plate tests correctly it is first necessary to be sure whether one is dealing with a normally pre-existing deflecting body or with one produced by immunization (see above). By means of combining experiments it must also be shown whether the deflection is caused by amoebocytes or amoebotoxins. It is not difficult, by binding them to the corresponding bacteria, to remove the amoebocytes produced by immunization. In most cases the addition of a moderate amount of bacteria care-

fully killed (65° for  $\frac{1}{2}$  hour) and centrifuged will suffice. In these cases, however, the supernatant fluid must always be examined macroscopically to make sure that all the bacteria have been removed by the centrifuging. For any such dead bacteria loaded with amoceptor, which should remain in the fluid, would serve to deflect complements in the further course of the experiment. However, in many cases it is possible to remove all the bacteria by centrifuging. In that case it is easy to show *that the bactericidal, as well as the complement deflecting power of the serum, has disappeared with the absorbed amoceptor*. If only the deflecting power of the serum remains, while the bactericidal power has disappeared, and if the comparative test has shown that one was not dealing with a normal anticomplement or such like, we conclude that a complementophile amoceptor is present, one which has originated from the amoceptor produced by immunization.

In many cases in which a plate test, as it has previously been described, has seemed unworkable, another method has been used to overcome the difficulty. Thus after allowing the immune serum to act, instead of pouring plates, one can take a loop from each test-tube and make slant agar streaks. If one then merely regards very broad results, such as no growth, luxuriant growth, one will obtain, by this simple means, useful comparative values. In this way Dr. Lapstein and I have several times determined the power of a gonococcus serum which we produced by immunization.

## XXXI. THE PROPERTY OF THE BRAIN TO NEUTRALIZE TETANUS TOXIN<sup>1</sup>

By Dr. E. MARX, Member of the Institute

WASSERMANN and Takaki's<sup>2</sup> communication stating that it is possible by means of normal brain substance to decrease the toxicity of tetanus toxin, or even, in suitable doses to entirely neutralize it, was undoubtedly of great theoretical and practical significance. Their statement was confirmed by many different investigators, Ransom,<sup>3</sup> Metchnikoff,<sup>4</sup> Marie,<sup>5</sup> Blumenthal,<sup>6</sup> Milchner,<sup>7</sup> Danyz,<sup>8</sup> Zupnik,<sup>9</sup> and others. These experiments were devised by Wassermann and Takaki as a test for the correctness of the side-chain theory, according to which the cells, susceptible to the poison, possess receptors which anchor the same. They argued, if the theory were correct, that the brain-cells which in vivo are susceptible to the poison should also be capable, at least in the fresh state, to bind the poison in vitro, i.e., it should be possible to neutralize solutions of tetanus poison with brain substance. As is well known the result of the experiments agreed with the theoretical premises and they were so interpreted by Wassermann.

This interpretation was first denied by Metchnikoff. He as well as Marie had repeated Wassermann's experiments and conceded

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<sup>1</sup> Reprint from the *Zeitsch. f. Hygiene und Infectious-Krankheiten*, Vol. 40, 1902.

<sup>2</sup> *Berl. klin. Wochensh.*

<sup>3</sup> *Deutsch. med. Wochensh.* 1898, No. 5 (communicated through v. Behring).

<sup>4</sup> *Annales de l'Institut. Pasteur*, 1898, pp. 81 and 263.

<sup>5</sup> *Ibid.*, 1898, p. 91.

<sup>6</sup> *Deutsch. med. Wochensh.* 1898, No. 12.

<sup>7</sup> *Ibid.*, 1898, No. 16.

<sup>8</sup> *Annales de l'Institut. Pasteur*, 1899.

<sup>9</sup> *Prager med. Wochensh.* 1899, Nos. 14 and 15.

their correctness, but on the basis of further experiments made by Marie, Metchnikoff was led to another interpretation of the results. Marie found that when poison and brain substance were injected separately, even large amounts of brain substance did not exert any protection. Metchnikoff, therefore, did not believe in any neutralization of poison by the brain substance in vitro. He saw the cause of the apparent neutralization in mixtures of tetanus poison and brain substance in the leucocyte-attracting power of the brain substance injected with the poison. According to him the leucocytes were the agents which destroyed the poison, and the brain substance only the means for attracting these.

It is hardly within my province to subject these experiments to a thorough criticism; that must be left to those directly interested. I should, however, like to mention two points which appear to me not to be sufficiently regarded. First, it must be remembered that with a dissolved antitoxin the success in neutralization on mixing antitoxin and poison in vitro is considerably higher than the therapeutic success which the same dose attains in an animal. In the above experiments there is added to this the fact that we are not dealing with a dissolved antitoxin. On the contrary, the poison-neutralizing power is exerted by a mass which, from experience, we know is absorbed with great difficulty.

Subsequently v. Behring, as a result of his combining experiments with brain substance, expressed doubts as to the correctness of Wassermann's explanation, without, however, positively taking either one side or the other. Basing his reasons on the experiments of Kitashima, v. Behring<sup>1</sup> stated his views as follows:

"If an emulsion of fresh brain substance from a guinea-pig is mixed with a certain dose of tetanus poison, a dose whose power is exactly known, it will be found that with small amounts the poison will completely lose its poisonous property; with larger amounts there is a distinct decrease of this property. One would now suppose that large amounts of poison, whose poisonous property has been decreased by means of brain emulsion, would require less antitoxin for their neutralization than before the addition of the brain emulsion. But this is by no means always the case. In the experiment—

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<sup>1</sup> v. Behring, *Allgemeine Therapie der Infectious-Krankheiten*, Part I, p. 1033.



0.008 cc. poison solution No. 3,  
0.2 cc. brain emulsion;  
one hour later:  
 $\frac{1}{1000}$  antitoxin unit —

we not only found no excess of antitoxin, but found that the injection of such a mixture into mice caused death by tetanus."

The result of this experiment led v. Behring to conclude that further study of the poison-neutralizing power of guinea-pig brain would probably decide the question in favor of Metchnikoff's views as outlined above. A subsequent study from v. Behring's institute demonstrated that a union evidently takes place when living brain and tetanus poison come together.

Ransom<sup>1</sup> studied the conditions found in the subarachnoid space after injections of tetanus poison or tetanus antitoxin. It would lead us too far to recapitulate these brilliant experiments, and I shall, therefore, content myself by quoting Ransom's conclusions which are as follows:

"These experiments strongly corroborate the assumption that tetanus antitoxin is bound in the central nervous system; they also indicate that this union takes place somewhat gradually."

There is surely no objection to our placing these experiments on the living brain parallel with those made on the dead brain. It would be incomprehensible for a brain, removed at once from a freshly killed animal, to be different in its property of binding tetanus poison from what it was a few minutes previously in the living animal.

I had just begun a study in this institute dealing with these problems, but discontinued them on the appearance of Ransom's paper since that had so well covered the subject.

Some time after this Kitashima's experiments were taken up by Gruber,<sup>2</sup> although without re-examination. In these experiments Gruber saw further proof of the incorrectness, according to him, of Ehrlich's Side-chain Theory. In response to this, however, Pallauf<sup>3</sup> very aptly demonstrated that a simple calculation will show that Kitashima's experiments cannot in any way be regarded as conclusive. He expressed himself as follows:

<sup>1</sup> Hoppe-Seyler's Zeitschrift für physiol. Chemie 1900-1901, Vol. XXXI, p. 282 et seq.

<sup>2</sup> Münch. med. Wochensch. 1901, Nos. 46-49.

<sup>3</sup> Wiener klin. Wochensch. 1901, No. 51.



"0.008 cc. tetanus poison No. 3 + 0.2 cc. brain; one hour later,  $\frac{1}{1000}$  antitoxin unit. Tetanus poison No. 3 is very powerful. 1 cc. equals 5 million mouse. 15 mouse is a fatal dose for a mouse; in the experiment, therefore, 40,000 mouse or more than 2600 - the fatal dose is employed, which quantity, to be sure, is neutralized by  $\frac{1}{1000}$  antitoxin unit. According to Wassermann, however, 1 cc. emulsion can at the most neutralize 10 fatal doses; according to others, from 30 to 100 fatal doses. Usually 1-5 cc. suffices to neutralize not over 20 doses of poison, an amount which is very minute when 2600 doses of poison are concerned."

It should also be mentioned that Blumenthal and Wassermann<sup>1</sup> opposed Gruber's view. Blumenthal called attention to the fact that when brain substance is added to a toxin solution it is possible by centrifuging to show that the original toxin solution has been robbed of its toxic power, a result which cannot be obtained with boiled brain. He also reminded his readers that he had shown how, by introducing the toxin *in vivo*, the power of the brain to neutralize poison had been diminished, as was seen on testing the same post-mortem. This diminution was due to the union of the brain substance with the toxin, and was in proportion to the amount of poison injected.

Wassermann too is still convinced that there is a chemical union. His view is also borne out by the fact that in the rabbit, in which, according to the researches of Donitz and Roux, an extensive distribution of receptors capable of binding tetanus toxin was to be assumed, other organs besides the brain are also capable of neutralizing the poison *in vitro*. This is in direct contrast to the guinea-pig in which only the brain possesses this power.

In view of all this we determined to finally decide whether on the addition of brain to tetanus poison there is an actual union of poison, and whether if this is so there is a summation of neutralizing actions of brain and antitoxin. Our old studies were therefore again taken up. We began with the re-examination of Kitashima's experiments, but under such conditions that the errors which, independently of us, Paltauf had already pointed out, namely, the employment of too large doses of poison, were avoided.

<sup>1</sup> Deutsche med. Wochenschr. 1902, Vereinsbeilage, No. 3.

## THE MATERIAL EMPLOYED, AND ITS PREPARATION.

In these experiments a great deal depends on the manner in which the brain emulsion is prepared. We shall therefore again describe the method in detail, although Wassermann and Takaki did so when they reported their experiments.

Each guinea-pig brain was thoroughly mixed with 10 cc. 0.85% salt solution. In order to obtain uniform and good results it is necessary that the emulsion be as fine as possible. For this purpose the brain substance was crushed and the salt solution added, at first drop by drop, until a fine uniform emulsion resulted. It is well instead of using a mortar to use conical glasses, such as are employed at the Rabies Inoculation Stations for preparing the fine cord emulsions for injections. These conical glasses are about 10 cm. high and taper not to a point, but to a hemispherical surface into which a ground-glass pestle fits.<sup>1</sup> This very fine emulsion is then forced through Herzberg funnels, such as are used in testing paper. If the emulsion is forced through the finest of these, fitted with wire-gauze with the smallest mesh obtainable, it will be found that the emulsion is actually free from macroscopically coarse particles.

The poison I employed was a tetanus toxin preserved in the institute for diagnostic purposes. This poison, I may add, owing to the special method of preparation, differed from Behring's test poisons (at least from those which can be obtained in the market) in being free from spores. This fact may perhaps not be without significance, for, under the conditions which here obtain, a development of the spores with consequent production of poison in the animal cannot be denied offhand.

This possibility must surely often be counted on. It was for this reason that Ehrlich long ago allowed only such tetanus poisons as were freed as much as possible from spores to be used for testing, and for exact experimental studies. I shall soon publish an account of the peculiarities of the procedure used in this institute for obtaining such poisons, and also describe a method for preserving tetanus poison permanently, which we have found very useful.

The antitoxin used was also that preserved for testing purposes. 1 gram. contains 100 A. E. *Behring*.

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<sup>1</sup> These can be obtained from F. and M. Lautenschläger, Berlin, N.

## METHOD OF MAKING THE EXPERIMENTS.

The method employed followed exactly in principle that employed by Kitashima. A 1 to 400 dilution of the normal solution of the poison was prepared. To each cubic centimeter of this, which represents forty times the fatal dose for a mouse of 15 gm., the desired number of doses of brain emulsion, or of a 1:10 dilution of this emulsion was added, the fluid made up to 2.5 cc. by the addition of 0.85% NaCl solution, and the mixture thoroughly shaken. At the end of an hour 0.5 cc. of the dilutions of serum in question were added and after once more thoroughly shaking,  $\frac{1}{2}$ -cc. doses of this mixture were injected subcutaneously into white mice weighing 15 gm. It may be mentioned that in the controls containing only brain and poison the procedure was exactly the same except that 0.5 cc. NaCl solution were added at the end of the hour instead of 0.5 cc. serum. The control containing only poison and serum was treated in exactly the same manner and was injected in the usual way after the antitoxin had been allowed to act in the toxin for thirty minutes. It may be added that no appreciable difference was observed if the mixture of poison+brain+serum was injected directly after the addition of the serum or if the serum was allowed to act on the brain+poison mixture for half an hour.

## RESULTS OF THE EXPERIMENTS.

My results, obtained from over two hundred experiments on mice, do not furnish the slightest ground for assuming that the phenomenon found by Kitashima is the rule. On the contrary, from my experiments I can positively conclude that there is always a summation of the poison-neutralizing action of the brain and antitoxin; furthermore that there is never any interference with the antitoxic action of the serum as a result of the previous action of the brain on the tetanus poison. This fact was constantly observed, no matter whether large or very small doses were employed. The series of tests with brain emulsions, as well as those with brain and poison alone without serum, do not, to be sure, proceed as smoothly as those with poison+serum; however, this is not at all surprising; on the contrary, it is quite natural that the particles suspended in the emulsion, even if they are very fine, cannot produce as uniform effects as a solution of antitoxin.

The results of my experiments were all the same and their significance is absolutely clear. From the large number of tests I shall therefore give but three. These will incidentally show the well known fact that the power to neutralize poison is often very different in different cases.

TABLE I.

Degree of Dilution of the Serum.	Control Toxin + Serum.	The Experiment. Toxin + 1.5 cc. Brain + Serum.
1:17500	† 3	moderately sick
1:15000	† 4	lightly sick
1:12500	† 4	" "
1:10000	† 7	moderately sick
1: 8000	† 9	" "
1: 6000	† 9	trace sickness
1: 4000	moderately sick	" "
Control: only toxin and 1.5 cc. brain	}	† 9

TABLE II.

Degree of Dilution of the Serum.	Control Toxin + Serum.	The Experiment. Toxin + 0.2 cc. Brain + Serum.
1:17500	† 3	moderately sick
1:15000	† 3	" "
1:12500	† 4	" "
1:10000	† 4	" "
1: 8000	very severely sick	" "
1: 6000	severely sick	trace sickness
1: 4000	moderately sick	" "
1: 3000	" "	well
1: 2000	trace sickness	" "
1: 1000	well	" "
Control: only toxin and 0.2 cc. brain	}	† 4

TABLE III.

Degree of Dilution of the Serum.	Control Toxin + Serum.	The Experiment. Serum I. Toxin + 0.1 cc. Brain + Serum.	The Experiment. Serum II. Toxin + 0.2 cc. Brain + Serum.
1:17500	† 4	very sick	very sick
1:15000	† 4	" "	" "
1:10000	† 5	" "	moderately sick
1: 5000	moderately sick	moderately sick	" "
Control: only toxin + 0.1 cc. or 0.2 cc. brain	}	† 4	† 4

All of these experiments show that the mice which received only toxin and brain died, whereas additions of antitoxin as did not by themselves suffice to neutralize the dose of poison were able to save the animals which received the doses of brain emulsion. Hence the action of the brain doses (which by themselves do not protect) adds itself to that of non-protecting doses of antitoxin and so forms a protective dose.

*Résumé.*

1. *The neutralizing effect possessed by guinea-pig brain on tetanus toxin is supplemented by that of antitoxin when these are allowed to act on the poison in vitro.*
2. *From this one can conclude that this neutralizing effect of guinea-pig brain on tetanus toxin and that of the antitoxin can be regarded as equivalent properties.*

## XXXII. THE PROTECTIVE SUBSTANCES OF THE BLOOD.<sup>1</sup>

By Professor Dr. P. EHRLICH.

MORE than ten years have passed since the studies of Flügge and of Buchner and of their pupils directed attention to the bactericidal substances present in normal blood serum and their relation to natural immunity. Buchner especially assumed that the serum of each animal species contained a simple definite protective body, the alexin, which was able to kill off *foreign* cells, especially bacteria and the blood-cells of other species; that this acts somewhat after the manner of a proteolytic ferment and leaves the cell elements of its own species unscathed. The recent development of the doctrine of immunity, inaugurated by v. Behring's discovery of antitoxin, has also shed considerable light on the nature of protective bodies preformed normally, so that it now seems advisable to subject the mutual relations existing between these to a closer analysis.

There can hardly be any doubt that, in accordance with the principle enunciated by Virchow for the relation existing between cell physiology and cell pathology, the normal protective substances are subject to the same developmental laws as the artificially produced antitoxic and bactericidal substances. It is obvious that with the artificially produced protective substances, especially with the antitoxins, it will be far easier to gain an insight into the mechanism of their development, for in this case one possesses not only the exciting agent (as, for example, the toxin), but also the resulting specific product (the specific antitoxin), making it possible to study their mutual chemical relations.

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<sup>1</sup> Address delivered in the general session of the 73d Congress of German Naturalists and Physicians, Hamburg, Sept. 25, 1901. (Reprinted from the *Deutsche med. Wochenschrift* 1901, Nos. 51 and 52.)

This, however, is not possible in the case of the substances naturally present, and, considering the complicated chemistry of the living organism, we shall probably long continue to be ignorant of the substances which act as the physiological excitants.

Hence it is not a mere coincidence that the attempt to formulate a theory for the development of the protective substances succeeded first in connection with those artificially produced. This is now well known as the side-chain or receptor theory. According to my view this theory is also of the highest significance for the conception of the nature of the alexins. I shall, however, first outline my views on this subject as they are applied to the formation of antitoxin, as this is comparatively the simplest to study.

There were, as you all know, chiefly two views concerning the formation of antitoxin, namely, the hypothetical metamorphosis of toxin into antitoxin, and the secretion theory, which approaches somewhat the side-chain standpoint. The former was based on the observation that the antitoxin excited by a certain toxin acts only against just this toxin and against no other. This specific action is such a conspicuous phenomenon that it was at first believed that the intimate relation of toxin to antitoxin could only be explained by assuming the toxin itself to be the mother substance of the antitoxin. So even to this day, Buchner maintains the view that the antitoxins and related substances do not correspond to preformed or even wholly newly formed constituents of the organism, but that they are non-poisonous transformation products of the substances introduced for purposes of immunization. In this case, therefore, the relationship of antibody to the substances exciting its production would be due to a *similarity* of the two components. In other words, there would be no antagonism such as exists between acid and base, but an attraction of like to like, as is seen, for example, in polymerization, in the attraction of crystallization, or in the structure of starch granules.

Against this I should like to point out that this assumption cannot apply even from a purely chemical standpoint because the processes advanced as analogous occur in concentrated solutions, while neutralization of toxin and antitoxin takes place in extremely dilute solutions.

The biological conditions, however, constitute the most serious objection to the assumption of a transformation of toxin into antitoxin. First comes the enormous difference in quantity which may

exist between the toxin introduced and the resulting antitoxin. Knorr, for example, has shown that the injection of tetanus toxin into a horse is followed by the production of an amount of antitoxin which would neutralize 100,000 times the dose of poison employed. Such an enormous disproportion cannot be reconciled with Buchner's view, according to which each part of toxin would make an antitoxin equivalent. This ratio can be explained only by a theory which makes the production of antibody more independent of the exciting agent.

Another fact, which cannot be reconciled with a transformation of toxin into antitoxin, is the marked difference existing between so-called active and passive immunity. If, for example, by injecting an animal with poisons or bacteria an active immunity is produced, this immunity may in favorable cases persist for years, while in passive immunity the preformed antitoxin introduced into the organism exists but a short time. Such a difference could not exist if the antitoxin were nothing else than transformed toxin, for in that case it should be absolutely immaterial how the antitoxin now in the organism had originated. The difference, however, depends on the fact that in active immunity the tissues of the body constantly produce new antitoxin, keeping pace with the excretion of the same.

This production of the antitoxin by the body-cells is furthermore confirmed by the interesting experiments of Roux and Vaillard, and of Salomonsen and Madsen. They took an animal which had been actively immunized, and whose serum showed a constant amount of antitoxin, and by means of repeated venesection abstracted a considerable portion of its blood. In case the antitoxin had been derived from the toxin introduced there should, now that the last traces of poison had disappeared from the body, have been a marked loss of antitoxin from the blood. On the contrary within a short time it was found that the amount of antitoxin had again reached its previous level. Another point in support of the assumption that the body-cells produce the antitoxin is an experiment of Salomonsen and Madsen, which shows that the amount of antitoxin present in the blood of an actively immunized animal is increased if the animal is treated with substances which increase the secretion of blood-cells in general, e.g. pilocarpine. This experiment was advanced by Salomonsen and Madsen as absolutely opposed to the transformation hypothesis and supporting their secretion theory.



There is one fact, however, by which the transformation hypothesis is especially refuted, namely, that antitoxins can occur in the blood of normal individuals. Thus diphtheria antitoxin is found in the blood of horses in about 20-30% of the animals examined, although diphtheria infection is surely a rare exception with these animals. Horse serum furthermore contains antibodies against one of the poisons produced by tetanus bacilli, tetanolysin, but not against the tetanizing poison of the same bacilli, the tetanospasmin, although the immune serum artificially produced contains both antibodies.

Just these observations, which can easily be extended, show that even the normal organism can produce true antitoxins without the intervention of the corresponding bacterial substances. Hence these antibodies cannot be transformation products of the poisons injected, but are products of normal cell activity. *The explanation especially of these normal processes constitutes one of the chief points in the side-chain theory.*

This theory is based primarily on a thorough analysis of the relations between toxin and antitoxin. It was found, by means of test-tube experiments with ricin and related bodies which act on red blood-cells, that it was extremely probable that toxin and antitoxin act chemically directly on each other, forming a new innocuous combination. It was now necessary to study the neutralization of these two substances in all directions in great detail. For this purpose I chose diphtheria toxin and antitoxin, because the guinea-pig organism furnishes such a uniform test object for this poison that exact quantitative determinations, such as are used in physics and chemistry, are attainable in animal experiments. The limit of error in the titration of diphtheria serum titrations is not more than 1%, surely an astonishing result if we consider that we are dealing with substances which chemically as yet are entirely unknown.

The results which I obtained in the earlier years of my investigations were really very discouraging, for they seemed to present an insurmountable obstacle for the chemical conception. In chemical processes when two substances unite to form a third substance, in accordance with the laws of stoichiometry, we must insist that these components act on one another in definite equivalent proportions. In the action of diphtheria antitoxin or toxin, however, this law seemed to be utterly disregarded. Thus in twelve different toxic bouillons I first determined the quantity which was neutralized by a constant amount of antitoxin; in certain instances by the official

standard unit of antitoxin. The figures thus obtained, as was to be expected, varied greatly: in one case the antitoxin unit neutralized 0.25 cc. toxic bouillon, in another case 1.5 cc. This is not in the least surprising, for it is well known that the amount of poison given off by the bacteria to the medium depends on the strain of the bacilli, on the preparation of the bouillon, etc., so that strong poisons and weak poisons arise. But, assuming that the toxin molecule follows chemical laws in its union with antitoxin, it was to be expected that in the different poisons the amounts neutralized by 1 I. E. (Immun Einheit = Immune Unit), and designated as  $L_0$ , would contain equal amounts of true poison, or in other words that the various poisons which differ in their  $L_0$  doses represent nothing more than more or less concentrated solutions of the same toxic substance. The amount of poison contained in a solution is measured in poison units, i.e., that amount of toxic bouillon which just suffices to kill a guinea-pig weighing 250 grm. in four days. Thus if in a certain poison *A* we find the amount neutralized by 1 antitoxic unit, i.e., the  $L_0$  dose, to be 1 cc., and if we further find that 0.01 cc. of the same poison suffices to kill a guinea-pig, we say that in this poison the  $L_0$  dose represents 100 poison units. In accordance with the law of equivalent proportions we should have expected that the  $L_0$  dose of the various poisons would contain the same number of poison units. As a matter of fact, however, the result was quite the reverse, for we found that the number of poison units contained in  $L_0$  varied from a minimum of 10 units to a maximum of 150. According to the view held at that time that the antitoxin was bound only by the toxin, this wide divergence from the laws of equivalence could not help but cause the assumption that the relations existing between these two opposing substances were other than purely chemical ones.

Finally by employing a method of study which has proved of considerable value in scientific investigations, namely, the genetic method, I succeeded in getting some light on this subject. Following this I subjected one and the same toxic bouillon to comparative tests at different times. I may be permitted to demonstrate this by means of a simple schematic example. In a freshly made poison we find that the quantity which is neutralized by 1 I. E., in other words the  $L_0$  dose, amounts to 1 cc., and that this contains 100 poison units. If the same poison is examined at the end of about six months, it is found that the  $L_0$  dose is the same, but that this contains only 50, i.e., half the number of toxic doses. That is to say, the toxic

bouillon still possesses the original neutralizing power but a weaker toxic action. Hence toxic action on an animal and combining power for antitoxin must be two different functions, the former remaining constant and the latter decreasing.

If we regard these conditions from the chemical standpoint, we shall see that they are most readily explained by assuming that the toxin molecule produced by the diphtheria bacilli contains two different groups, of which one, termed the haptophore group, effects the union with antitoxin, while the other, the toxophore group, represents the actual cause of the toxicity. These two groups also differ in their stability, for the toxophore group is very unstable, the haptophore group far more stable. Modified poisons in which there has been a destruction of the toxophore group while the haptophore group has been preserved, and which have therefore completely lost their toxic action, are called "toxoids."

The presence of such toxoids fully explains the apparent deviations from the laws of equivalence which are observed in neutralizing tests with toxin and antitoxin. This furnishes new and, to my mind, incontrovertible proof for the chemical view of the process of neutralization.

In diphtheria poison at least, for reasons into which I cannot here enter, it seems that the affinity of the haptophore group of the toxoid molecule for the antitoxin is exactly the same as that of the unchanged toxin. This indicates that the two functioning groups of the toxin molecule possess a certain degree of independence. I have tried further by means of refined investigating methods, such as partial neutralizations, to extend the views concerning the constitution of the poison molecule. My observations, so far as the facts are concerned, have been completely confirmed from various sources. Mention should be made especially of the excellent study of Madsen on diphtheria toxin and tetanus toxin, and of the interesting experiments recently published by Jacoby on ricin and its toxoids.

In studying the two groups of the poison molecule, we are concerned not only with a satisfactory explanation for the process of neutralization. The presence of these groups gives us an insight both into the nature of the poisoning and the origin of the antitoxin.

So far as this last point is concerned, two facts in particular indicate that the haptophore group takes a leading part in the immunity reaction in the organism, viz., (1) the observation that

toxoids, which lack the toxophore group, are still capable of exciting the production of typical antitoxins, and (2) that toxins whose haptophore group is preoccupied by antitoxins lose, as a result of this procedure, their power to produce antitoxins. Now in order to understand the essential rôle played by the haptophore group in the formation of antitoxins and of the antibodies in general, it is necessary above all to study the other side of this question, namely, *the functions of the living organism in the formation of antibodies*.

The demonstration that it is the haptophore group of the toxin molecule that excites the production of immunity leads us at once to regard the process of assimilation of the living cells as most important in our study. Since the beginning of medicine it has been, and still is, generally accepted that chemical substances can act only on those organs with which they are capable of entering into direct chemical relations. In his "Cellular Pathology," Virchow expressed this view in his usual clear and forcible manner: "Just as the single cell of a fungus or an alga abstracts from the fluid in which it lives as much and the kind of material as it needs for its vital processes, so also the tissue cell within a compound organism possesses elective properties by virtue of which it disregards certain substances and takes up and utilizes others."

"We also know that there are a number of substances which have a special attraction for the nervous system when introduced into the body; that even among this group there are substances which possess intimate relations to certain particular parts of the nervous system, some to the brain, others to the spinal cord or to the sympathetic ganglia, a few to certain special parts of the brain, cord, etc. I may mention morphine, atropine, curare, strychnine, digitalin. On the other hand we know that certain substances are intimately related to certain organs of secretion, that they permeate these secreting organs with a certain selective action, that they are excreted by them, and that when supplied in excess such substances cause an irritation in these organs."

It is remarkable that this axiom was not re-evaluated in the development of scientific pharmacology, and that only within the last ten years, thanks to the labors of Hofmeister, Overton, Spiro, Hans Meyer and myself, an improvement has taken place in this respect.

According to these newer researches there is not the least doubt that the causes of this elective lodgment in certain cell domains are not all of the same nature. In general the modern pharmacological

school now believes that the substances ordinarily foreign to the organism, such as the indifferent narcotics, alkaloids, antipyretics, antiseptics, do not effect a firm chemical union with the body elements, but that their distribution follows the laws of solid solutions or of the formation of a loose salt. In the case of the poisons acting on the central nervous system it is especially the fat-like substances of the nerve tissue, the so-called lipoids, which take up the narcotics, just as ether takes up the alkaloids in the Stas-Otto procedure of detecting poisons. There are a number of reasons in support of the view that the pharmacological agents in question are stored up unchanged in the cells or in certain constituents thereof, especially in those similar to fat.

Naturally this does not deny the possibility that certain substances foreign to the body may enter an albumin molecule by substitution. Thus if protoplasm is treated with nitric acid the nitro group enters the albumin radicle, giving rise to a yellow color. Such substitutions, however, in the conditions under which pharmacological actions can occur, will usually only be effected by combinations possessing high internal tension and for that reason capable of such addition reactions. This may perhaps be the case with vinylamine, which, according to Levaditi's experiments conducted in my laboratory, produces necrosis of the renal papillæ in a large number of animals, a phenomenon probably to be ascribed to such a chemical anchoring.

The ordinary medicinal substances, however, are not so constructed that they can produce such energetic actions. In general we may assume that chemo-synthetic processes do not play a prominent part in their distribution.

It may, however, be regarded as an absolute fact that synthetic processes play an important rôle in the life of the cell in another direction. If by boiling certain cell material with acids we are able to split off certain definite groups (such as those of sugar, etc.), this fact proves the chemical character of this combination. As a matter of fact the two series of phenomena which we are here dealing with have long been separated by general custom. The term *assimilability* is reserved exclusively for those substances which are anchored by the cells *synthetically*, and which in this way become constituents of the protoplasm. No one would think of speaking of morphine, or of methylene blue, substances which enter into certain cells and lodge there, as being assimilable.

These explanations will suffice to show that the term assimilability, as I employ it, is restricted somewhat more than is customary, for I reserve it exclusively for the specific nutritive substances of the living protoplasm. According to this view the process of cell assimilations is a synthetic one which presupposes the presence of two groups effecting the synthesis and having a strong *chemical* affinity for each other.

Hence I assume that the living protoplasm possesses side-chains or receptors which possess a maximum chemical affinity for certain particular groups of the specific nutritive substances, and that they therefore anchor these substances to the cell. The receptor apparatus of the cells is highly complicated, the red blood-cell, for example, possessing perhaps a hundred different types of receptors.

If this view is accepted and it is recalled that in the toxin molecule it is the haptophore group which effects the development of immunity, only a very small step is required in order to gain an insight into the nature of antitoxin formation. This is the very natural assumption that among the various receptors—perhaps by chance—the haptophore group of the toxin finds one which possesses an especial affinity for this haptophore group. It is not at all necessary that every bacterial toxin should find fitting, i.e. toxophile, receptors in every animal species. On the contrary just this absence of receptors constitutes one of the reasons why certain animal species are immune against certain particular poisons. Furthermore, all the facts indicate that the susceptibility, i.e. the receptiveness, of an organism for a certain toxin is associated with the presence of such toxophile groups of the protoplasm, a point which finds suitable expression in the term *receptors*.

As a result of anchoring the toxin molecule by means of the haptophore group the cell is influenced in two directions. Primarily, owing to the lasting influence of the toxophore group, it sickens, a condition which manifests itself by disturbed functions and possibly by pathological anatomical changes. Besides this, however, in a manner shortly to be discussed, a regenerative process is begun which can lead to the formation of antitoxin. Since this regenerative process can be excited by toxoids lacking the toxophore group, as well as by the toxins themselves, we must assume that it is intimately related to the haptophore group. Hence the two parallel processes, antitoxin production and toxic action, are independent in that they are caused by two different groups. In harmony with this

is the fact that the two processes may interfere with one another; a marked pathological action can diminish the regenerative process or even prevent it entirely. This is shown, for example, by the fact that it is almost impossible in the case of certain animals highly susceptible to tetanus poison, such as mice and guinea pigs, to produce antitoxin by means of unmodified poison, while the result is easily attained by the use of toxoids.

Coming now to the regenerative process, which leads to the production of antitoxin, it will be found by any one familiar with the fundamental principles formulated by Carl Weigert that there is nothing remarkable about the process. The receptor which has anchored the haptophore group of the toxin or toxoid molecule becomes useless for the cell because of this occupation; it is no longer able to exercise its normal function, namely, the anchoring of nutritive substances. The cell has thus suffered a loss which must be replaced.

In such processes it is very common to find, as Weigert's researches have shown, that the loss is not merely *replaced*, but that it is *overcompensated*. The same thing takes place in the methodical immunization when continued and ever increased doses of immunizing substance are introduced. Part of the newly formed receptors still attached to the cell are occupied by the immunizing substance only to be replaced by a regeneration greater in degree than before. Owing to this increased demand the protoplasm to a certain extent is trained in one direction, namely, to produce anew a certain kind of constituent, the receptors in question. Finally, such an excess of receptors is produced that there is no longer room in the protoplasm for them. Then they are thrust off as free molecules and pass into the body fluids. According to this view the antitoxin is nothing more than the thrust-off receptor apparatus of the protoplasm, i.e., a normal cell constituent produced in excess.

From among the many facts already at hand I shall select merely a few to serve as proof of the correctness of this hypothesis, this "side-chain theory," as it is called.

The first point deals with the demonstration in normal tissues of the toxinophile receptors assumed by the theory. Although such an anchoring of the poison by the organs had already been demonstrated by the clinical course of the poisoning and by Dönitz's therapeutic experiments on animals poisoned with tetanus and diphtheria poisons, it remained for Wassermann to show that certain body



elements anchor the toxin even in a test-tube and neutralize the toxin just as does the antitoxin. If he added crushed fresh guinea-pig brain to tetanus toxin, he found that the brain substance anchored the toxin in such a manner that not only was the supernatant fluid robbed of its toxic action, but that the brain laden with tetanus toxin also exerted no toxic effect. From this we can conclude that a chemical union has taken place between constituents of the ganglion cells and the tetanus toxin. This combination is so firm that it is not broken up on being introduced into the animal body; as a result the toxin remains innocuous.

That this is really a specific reaction and not, for instance, merely an absorption is shown by the fact that boiled brain, in which the chemical groups in question are destroyed, is just as little able to exert this action as the pulp of any other organ of the guinea-pig.

In addition to this Ransom has shown that the brain of living animals possesses the same toxin-destroying power. In view of this it would appear that the objections made by Danysz, which refer to the divergent behavior of the decomposed brain pulp, possess no great significance. I will not deny the fact that the favorable result achieved in tetanus is evidently due only to the coincidence that the tetanophile receptors are present in large quantity in the brain. Such a coincidence, of course, need not obtain for every poison. If the organs endangered by the toxin contain only small quantities of toxin receptors it will be found that with what are, at best, very coarse experimental methods these receptors escape detection. This is the case, for example, with botulism toxin and diphtheria toxin.

Such confusing chance occurrences can, however, be avoided with certainty if one employs poisons artificially produced, poisons which, owing to their mode of production, are directed against certain particular kinds of cells. The hæmolysins produced by injections of blood, spermotoxins, and numerous other cytotoxins may serve as examples. In all of these cases it can positively be proved that the toxin is anchored by the susceptible cells in specific fashion.

The second point concerns that premise of my theory which states that the same organs which possess a specific affinity for the poison molecule are able to produce antitoxin. In this connection the very neat experiments made by Römer on abrin immunization should be mentioned. As is well known, abrin, the toxalbumin of jequirity beans, is able to excite marked inflammation of the con-



conjunctiva in man and animals. I have shown, furthermore, that it is possible, by means of conjunctival instillations, to actively immunize rabbits against abrin. Romer immunized a rabbit by means of rapidly increased doses into the right eye and killed the animal at the end of three weeks. It was then found that the conjunctiva of the right eye which had been the site of the inflammatory process was able, when ground up with a suitable amount of abrin, almost completely to neutralize the action of this poison, whereas the other conjunctiva, when similarly ground up with abrin, was unable to protect the animal from death. From this Romer rightly concludes that in this conjunctival immunization part of the antitoxin is furnished by the conjunctiva which reacts locally. Aside from its theoretical interest I believe that this demonstration of the local origin of antitoxin at the site of injection possesses great practical significance. In certain cases the possibility is thus given to transfer part of the antitoxin production from the vital organs to the indifferent connective tissues.

The third point concerns the thrusting-off of the surplus receptors. A prerequisite for this thrusting-off is that the receptors in question, which are normally firmly attached to the protoplasmal molecule, become loosened. In several favorable cases it has been possible to confirm this postulate of my theory experimentally, though to be sure these deal with immunization by bacteria and not with soluble poisons. Pfeiffer and Marx succeeded in showing that with a suitably conducted cholera immunization it is possible to find a period at which the blood is still free from protective substances, although the specific protective substances can be abstracted from the blood-forming organs by crushing them up with salt solution. In my opinion this can be due only to an extraction of receptors which, since it is just previous to their extrusion, are only loosely attached to the protoplasmal molecule.

Almost simultaneously with Pfeiffer and Marx, the same results were obtained by Wassermann with typhoid, and these were later confirmed by Deutsch. In all of these experiments the hematopoietic system represents the site of production of these antibodies. The significance of this circumstance for the immunizing process has been pointed out by Metchnikoff's teachings.

These few examples will suffice to show that the side-chain theory has fully stood the test of experiment. During the many years of my experimental activity I have not met a single fact which con-

tradicts this theory and might serve to refute it. I may, therefore, regard the theory as well established and proceed to discuss in detail several important points which follow from it.

The side-chain theory explains in the most natural fashion the specific relations existing between toxin and the corresponding antitoxin. Furthermore the theory makes the immunizing action of the antitoxins perfectly comprehensible. When injected subcutaneously into animals in the usual manner the poisons are brought to the organs possessing toxinophile receptors (susceptible organs) by means of the circulation. If, however, these poisons meet with free toxinophile groups in the blood, they will at once combine with the same and so be diverted from the susceptible organs. A Behring has expressed this hypothesis as follows: "The same substance which when in the cells is a prerequisite and cause of the poisoning becomes the healing agent when present in the blood."

To my mind we are here dealing with a general biological law which is not limited to the toxins but applies to a great many, if not to all, poisonous substances. I need only cite the saponin poisoning of red blood-cells. Ransom found that the blood-cells take up saponin owing to their content of cholesterol and are, as a result, subjected to the deleterious action of the poison, whereas certain sera, which exert a protection against saponin poisoning *owe this protective property to the same cause, namely, the presence of cholesterol in the serum.*

Furthermore the theory at once explains the fact that the tissues of an immunized animal are subject to the action of the poison when in some way the action of the antitoxin contained in the serum is prevented. Thus Roux showed that rabbits immunized against tetanus become poisoned just as rapidly as control animals if the tetanus poison is brought into direct contact with the brain-cells by means of intracerebral injections. This fact is demanded by my theory, for, just as in immunized animals, the ganglion cells contain an excess of toxinophile groups and are thus especially adapted to anchor the poison which injures them. It was a grave error on the part of Roux to suppose that this experiment controverted the side-chain theory. Roux thought that according to my view a considerable amount of antitoxin had accumulated in the brain-cells and that therefore the immunized animals should possess a local brain immunity. There is evidently a misconception as to the term "antitoxin." Just as we cannot term any mass of iron a lightning-rod,

but restrict this term to such masses of iron which deflect the lighting from a particular point, so we must restrict the term antitoxin to those toxinophile groups which circulate in the blood and thus deflect the poison from the susceptible organs. The toxinophile groups present in these susceptible organs are not *toxin deflectors* but *toxin attractors*.

The theory also explains why the property of producing antitoxins is restricted to certain products of metabolism of living cells. All experiments to produce antibodies by means of chemically well defined toxic substances, such as morphine, strychnine, saponin, etc., have failed.

If we bear in mind that the distribution of these substances in the organism takes place without chemical union and therefore without the intervention of receptors, the negative result of these experiments will not surprise us. The property of forming antitoxin is possessed only by such substances as possess a group able to unite with the side-chains or receptors which effect assimilation. It must be remembered that all the poisons which excite the production of antitoxin are highly complex products of animal and vegetable cells, which in their chemical properties approach the true albumins and peptones. In 1897, by means of my theory, the production of antitoxin and the binding of foodstuff were first brought into connection. At that time nothing was known of the fact that even *ordinary foodstuffs* are capable of an analogous action. I have therefore been able to regard as an agreeable confirmation of my views the circumstance that this consequence of my hypothesis has actually repeatedly been demonstrated within the past year, especially by Bordet.

If animals are injected with milk, it is found that their serum gains the property of precipitating the milk in curds. This precipitation is also strictly specific, since numerous experiments show that the coagulating serum obtained by treatment with goat milk coagulates only goat milk, and not the milk of other species, as, for example, that of women or cows.

The results are similar if animals are injected with other albuminous substances, e.g., with the sera of different species or with egg albumin. In this case in the serum of the animal there develop substances (termed coagulins or precipitins) which specifically precipitate the corresponding kind of albumin.

Deviations from the law of specificity occur only in so far as the sera of closely related animal species contain substances more or less similar. Thus

the coagulin obtained by testing rabbits with human serum precipitates only human serum and the serum of the nearest related species, apes. This reaction, which was developed especially by the researches of Eblenbuth and of Wassermann, was therefore proposed for the further identification of blood.

From this we see that, entirely in harmony with my views, the injection of foodstuffs is followed by the production of typical antibodies, which combine with the exciting agent in a specific manner. An analogous reaction takes place in the normal processes of cell nutrition and serves as the chief source of the protective substances present in normal blood in such great numbers.

The conditions become much more complicated than those just described if, instead of the relatively simple soluble metabolic products, living cell material is employed. This is the case, for instance, in immunization against cholera, typhoid, anthrax, erysipelas of swine, and many other infectious diseases.

In these diseases under certain circumstances there develop many other reactive products beside the antitoxins produced against the bacterial toxins. The reason for this is that every bacterium is a highly complex living cell which, when it disintegrates in the animal body, gives rise to a large number of different components. Of these a great many are able to produce antibodies.

Hence as a result of the introduction of bacterial cultures, in addition to the specific bacteriolysons, which cause a solution of the bacteria, we see substances develop, such as the antileptinins (v. Dungern, Morgenroth, Brodt), the much discussed agglutinins (Gruber, Durham, Pfeiffer), and the coagulins (Kravis, Bordet), which specifically precipitate certain albuminous substances that have passed into the culture fluid.

The most interesting and important of the substances arising in such an immunization are undoubtedly the bacteriolysons, which have been studied especially by Pfeiffer and Bordet. At first it is highly surprising that the injection of cholera vibrios into the animal body should be followed by the formation of a substance which is able to dissolve the cholera vibrio, *and only this bacterium*. This action is so perfectly adapted to the purpose and is apparently so novel that it seems to fall beyond the pale of the normal functions of the body. It was therefore of the highest importance to explain, from the standpoint of cellular physiology, the origin of these substances also. The solution of this problem offered considerable diffi-

culties and did not succeed until the haemolysins were used in the experiments in place of the bacteriolysins.

Haemolysins are peculiar poisons which destroy red blood-cells. Such haemolysins are found in part in certain normal species of serum, in part they can be produced artificially, as will be subsequently described. In their fundamental properties they correspond entirely to the bacteriolysins, but possess the great advantage over the latter in that they readily permit the employment of test-tube experiments whereby the individual variability of the animal body is excluded, and so allow accurate quantitative determinations.

Belfanti and Carbone discovered the curious phenomenon that the serum of horses, after they had been treated with blood-cells of rabbits, contains substances which are highly toxic to rabbits, and only to these animals. Bordet showed that the cause of this toxicity is a specific haemolysin directed against the rabbit blood-cells. He showed further that such haemolysins, derived by injection of foreign blood-cells, lose their power to dissolve blood when heated for half an hour to 55° C. Bordet found also that the haemolytic property of such inactivated sera is again restored if certain normal sera are added. These important observations showed a complete analogy between these phenomena and those observed with bacteriolysins by Pfeiffer, Metchnikoff, and especially by Bordet. In the case of bacteriolysins it was found that serum freshly drawn from a goat immunized against cholera is able to effect solution of cholera vibrios, i.e., to give the so-called Pfeiffer reaction. Apparently this property disappears spontaneously if the serum is allowed to stand; it disappears rapidly when the serum is heated to 55° C. The cholera serum rendered inert by heating exerts its protective power in the animal body unchanged, and in test-tube experiments it attains its original solvent power on the addition of small amounts of normal goat or guinea-pig serum, although the latter do not by themselves injure cholera vibrios.

These experiments show that in bacteriolysis two substances act together; one, contained in immune blood, is relatively stable and represents the carrier of the specific protective action; the other, present in every normal serum, is easily destroyed. For the present the former is called the "immune body," while the latter, since it complements the action of the immune body, is called the "complement."

Since the haemolysins are by far the most convenient for experi

mental study, Dr. Morgenroth and I have endeavored in these studies to cover the mode of action of these two components on the susceptible object, the red blood-cells. For this purpose we first prepared solutions containing either only the immune body, or only the complement. These solutions were then brought into contact with the appropriate blood-cells, after which the fluid and blood-cells were separated by means of the centrifuge. The two portions were then tested to determine whether these substances had been taken up by the blood-cells. These experiments showed that the blood-cells are incapable of taking up complement alone, whereas they eagerly take up the immune body. If, however, the serum contains both components, they are both bound by the blood-cells in question.

A confirmation of this fact was furnished by Bordet, who showed that blood-cells or bacteria which by previous treatment have become loaded with immune body, abstract the complement from fluids containing the same with great avidity. These facts have been confirmed from all sides. They show that the blood-cells, or the bacteria, anchor the immune body but not the complement, but that the complement is also bound as soon as the immune body has been anchored.

Morgenroth and I have made these relations more easily comprehensible by means of the following assumptions concerning the constitution of the immune body and complement.

We believe it necessary to assume that the immune body possesses *two* kinds of haptophore groups, one of high affinity which combines with a corresponding receptor group of the red blood cell or bacterium, the other a group of less affinity which combines with the complement exerting the deleterious action on the cell. Hence the immune body is a kind of intermediate element which links complement and red blood-cells. In order to denote this function I have proposed the name "amboreceptor," which is to express this two-sided grasping power.

According to our conception the complement possesses a constitution analogous to that of the toxins. Thus it possesses a haptophore group which effects the specific combination with the amboreceptor. The presence of this is confirmed by the existence of analogues of antitoxins, namely, corresponding anticomplements. Besides this the complement possesses a second group, the cause of the injurious action, which is analogous to the toxophore group of the toxins. In view of the properties of this group, partly toxic, partly ferment-like, I have decided to name it the "zymotoxin" group. If one cares

to illustrate the action of the two components by means of a crude comparison, the action of gun and cartridge may be taken. The complement in itself is harmless, like a cartridge, which only acquires destructive power by being introduced into the gun. In like manner only by the exclusive mediation of the amboceptor is the injurious action of the complement called forth and transmitted to certain particular elements.

In opposition to this conception Bordet maintains the view that complement and immune body do not combine as we believe, but that the entrance of the immune body into the cell substance exerts a specific injury to the latter, an injury which manifests itself by the fact that now the cells succumb to the action of the simple protective substance present in blood serum, namely, Buchner's "alexin."

In other words, by means of the immune substances the blood-cells are made susceptible, "sensitized," to the action of the alexin. In conformity with this Bordet terms our immune body or amboceptor the "substance sensibilatrice" and our complement the alexin.

Although this view is also shared by Buchner, there are many reasons why I cannot accept it, especially in view of the observation made by M. Neisser and F. Wechsberg concerning the peculiar phenomenon of deflection of complement through an excess of immune body. To begin it is absolutely impossible to picture to one's self the nature of this sensitization. If Bordet believes that the sensitizer acts after the manner of a safety-key which, when introduced into a particular lock, makes the introduction of a second key possible, I must say that I cannot understand this comparison. It can positively be proven that the red blood-cell possesses no complementophile groups, since neither in the normal state nor after death does it lay hold of complement. The living blood-cell, as well as that killed by heating, however, through the occupation with the immune body, acquires the property to anchor complement. It surely is much more natural to believe that the immune body itself, the amboceptor, is the carrier of the group which binds the complement, than to assume that new complementophile groups arise owing to the action of the sensitizer. Finally, one can conceive of such a process in a *living* cell, one therefore capable of alteration, but in the case of *dead* cells which have been treated by heat or all sorts of chemicals, in the case of stabilized albumin as one might say, this assumption cannot be allowed.

Bordet's assumption furthermore does not explain the fact that

an immune body derived from a particular species is most surely activated by the serum derived from the same species. From the standpoint of Bordet's theory it would be most puzzling to understand why an anthrax immune body derived from a sheep should sensitize the bacilli against just the sheep alexin, one derived from a rabbit against just the rabbit alexin. From the standpoint of the amboceptor theory, however, such a phenomenon does not offer the least difficulty, since it is natural that the amboceptors circulating in every animal species are fitted to their own complements.

I wish to mention still one more point which plays a great rôle in Bordet's views. Bordet assumes that the alexin is a simple [einhheitlich] substance, whereas I maintain that there is a plurality of complements. Some very interesting experiments have recently been published by Bordet which appeared to support the unitarian view.

He first determined that a certain serum, e.g. guinea-pig serum, was able to activate two different immune bodies, e.g., a cholera-immune body and a hæmolytic immune body. To this guinea-pig serum Bordet added sensitized blood-cells, i.e., blood-cells eager to take up, and susceptible to complement. If now he waited until hæmolysis had begun, he found that the guinea-pig serum had lost its property to dissolve sensitized cholera vibrios. The same thing occurred if he reversed the experiment.

Although it was easy to confirm the experiment of this distinguished investigator, I found it impossible to accept Bordet's conclusions. This experiment is only then positive proof for a simple alexin (in this case for the identity of bacteriolytic and hæmolytic alexin) if it can be shown that the two immune bodies in question are acted on by only a single complementophile group and not by a plurality of such groups. Previous investigations, however, have shown that the immune sera artificially produced are not simple in character but are made up of a number of different amboceptors possessing different complementophile groups.

Nevertheless I consider Bordet's experiments so important that I have once more had this question thoroughly studied by Dr. Sachs and Dr. Morgenroth. These gentlemen were able to establish positive proof for the existence of different complements. Dr. Sachs, for instance, studied these conditions in goat serum, employing for the purpose five different combinations of immune body, each of which could be complemented by goat serum. If goat serum



contained only a single complement, the course of the five series of tests should have been identical when the complement was affected. It was found on the contrary that under the influence of digestion, for example, one completion remained intact, while four others disappeared. By means of absorption further analogous differences were manifested which made the assumption certain that in this case four different complements come into action. Since these results positively prove the existence of a plurality of complements I think it will be unnecessary here to bring forward additional evidence in support of this.

A résumé of these observations confirms my view that the mechanism of hæmolysis and bacteriolysis is most easily explained by the amboceptor theory.

So far as the origin of the two components which take part in this reaction are concerned there is not the least doubt that they are of cellular origin.

I assume that, in addition to the ordinary receptors which serve to take up relatively simple substances, the cells contain higher kinds of receptors designed to take up large-moleculated albuminous substances, as, for example, the contents of living cells. In this case, however, the fixation or anchoring of the molecule constitutes only a prerequisite for the cell's nutrition. Such a giant molecule in its natural state is useless for the nutrition of the cell and can be utilized only after it has been broken down into smaller constituents by fermentative processes. This will be accomplished most readily if the grasping group of the protoplasm is also the carrier of one or several fermentative groups which will immediately come into close relation with the molecule to be assimilated. It seems as though the economy of cell life finds it advantageous for the required fermentative groups to come into action only temporarily, perhaps only in case of need. This purpose is effected most simply if the grasping group possesses another haptophore group which can anchor the ferment-like substances present in the serum, the complements. Hence such a receptor of the higher order possesses two haptophore groups of which one anchors the foodstuff, while the other is complementophile. It is obvious that when, as a result of immunization, such receptors reach the blood, they will exhibit the properties which we have found to belong to the receptor type.

In regard to the second constituent, the complements, we shall not err if we regard these as simple cell secretions, designed to serve

internal metabolism. In accordance with the conception of Metchnikoff we must for the present believe that the leucocytes are primarily concerned in their production.

From these points of view the organism's immunity reaction loses the mysterious character which it would have if the protective substances artificially produced represented a constituent originally foreign to the organism and to its physiological economy.

But we have seen that immunity represents nothing more than a phase of the general physiology of nutrition, a view in which I agree entirely with that distinguished investigator Metchnikoff. Phenomena entirely analogous to those of the formation of antibodies are constantly occurring in the economy of normal metabolism, in all kinds of cells in the organism the absorption of foodstuffs, or of products of intermediate metabolism, can lead to the formation or the thrusting-off of receptors. Considering the large number of organs and the manifold chemistry of their cells it need not be surprising that the blood, which is representative of all the tissues, contains an innumerable number of such thrust-off receptors. To these I have given the collective name of "haptins." Only in recent years, thanks to these very theoretical considerations, have we reached a point where we can get some idea of this enormous multiplicity.

In addition to the true ferments and those ferment-like substances, the complements, already mentioned, the blood normally contains a number of substances which act specifically against certain substances present in solution.

Chief among these I may mention the normal antitoxins, and as examples of these the diphtheria antitoxin and antitetanolysin of normal horse serum, the antistaphylo toxin of normal human serum, and the anticrotin of pig serum. Next come the antiferments, such as antirennin, antithrombase, anticynarase, and others. We also normally find substances which prevent the action of specific hæmolysins and bacteriolysins, being directed in one case against the amboceptor, in another against the complement. For example, in goat blood I discovered an antiamboceptor which was directed against a goat-blood hæmolysin obtained in accordance with Bordet's procedure. In the blood of one animal species P. Müller of Graz found antibodies directed against certain complements of other species of animals, and which may, therefore, be termed normal anticomplements.

Of still greater interest, however, are those haptins which are

directed against living cells of all kinds, thus, against vegetable cells, such as bacteria, and against animal cells, such as red blood-cells, leucocytes, spermatozoa, epithelia, and others. The haptins which are so antagonistic to cells are divisible into two large groups: (1) the agglutinins, which cause the bacteria or other cells to stick together, and which through the researches of Gruber, Durham, and Widai have attained such great diagnostic significance; (2) the bactericidal or cytotoxic substances, and these are intimately related to natural immunity. In case the substances not only kill but also exert a solvent action we call them lysins, and speak of hæmolysins, bacteriolysins, etc. Thus a certain blood serum, e.g. dog serum, will simultaneously exert antitoxic, anti-fermentative, agglutinating, bacteriolytic, and cytotoxic effects against the appropriate substances. If we consider one of these functions by itself, e.g., the agglutinating function of a certain serum, we shall be met with the question whether or not this property is due to one simple substance, the agglutinin. Numerous experiments have shown that this is not so, but that in this precipitating process just exactly as many different agglutinins take part as there are present different agglutinable substances. It is easy to demonstrate this plurality by means of the principle of specific union introduced by me.

If, for example, a certain serum is able to agglutinate two varieties of blood-cells, say rabbit and pigeon blood-cells, and two kinds of bacteria, as cholera and typhoid, it should be found, in case this plural effect were produced by a single simple agglutinin, that absorption by one of these elements, e.g. the cholera vibrios, would remove the other three actions also. As a matter of fact, however, the serum which has been shaken with cholera vibrios, while it will no longer agglutinate cholera vibrios, is still able to produce agglutination in the other three elements, and vice versa. In this case, therefore, four different agglutinations take part.

Results entirely analogous to these are obtained if the other functionating groups contained in blood, e.g. the antitoxic, bacteriolytic, etc., are examined in a corresponding manner. These facts confirm the pluralistic view first maintained by me, according to which every blood serum contains many hundreds, or even thousands, of effective haptins. All of these, with the exception, perhaps, of ferments and complements, owe their origin to an excessive assimilative metabolism. Their peculiar action on certain substances foreign to the body may be regarded as due to an incidental meeting. To a large extent, therefore, they are to be looked upon

as luxuries which are not in themselves of any significance for the life of the organism. Of what use is it to a person or to an animal to have circulating in his blood a great variety of substances directed against heterogeneous materials which under normal circumstances never come into account, and which at the most are brought into relation with these substances only by the experimenter? Of what use is it to a goat to have in its blood certain substances which are directed against the red blood cells or the spermatozoa of other animals, since these do not normally get into the circulation? Furthermore every experimenter finds that the blood serum is subject to constant change in most of its haptins, a fact which argues strongly against the assumption that all of these substances in a *free* state play an important or even necessary rôle in the organism.

I cannot and do not deny that with such a superabundance of combinations in every serum substances will also be present which either by themselves or in conjunction with complements are able to destroy invading injurious bodies, especially bacteria. These substances then may be regarded as acting as defensive agents. In spite of this, however, I believe it is wrong to group this most complex system of haptins under the collective name alexin, because this leads to an incorrect unitarian view which cannot help scientific progress. These remarks are in no way intended to detract from the very valuable work of Buchner; his study on alexins, viewed in the light of that time and according to the then state of science, must be regarded as a masterpiece which has been of enormous value in the development of this subject.

Still another difference of opinion existing between Buchner and myself concerns the bactericidal and haemolytic power of normal blood serum, and these properties Buchner again ascribes to the action of his alexin conceived as a simple substance. In opposition to this I have demonstrated that the conditions in normal hæmolysins are exactly the same as in the artificial hæmolysins, for here again two different components act together, one of them is thermostable while the other corresponds to the complements. This fact has been confirmed by a large number of observers among whom I may mention v. Dungern, Moxyer, London, P. Muller, Meltzer. All these authors, like myself, have come to the conclusion that the thermostable substance necessary for the lytic process corresponds in every way to the artificially produced immune bodies or antio-

ceptors. The haemolysins occurring naturally and those artificially produced manifest their action according to exactly the same mechanism. According to the observations of Pfeiffer and of Moxter, as well as to certain experiments of Wechsberg and M. Neisser, still to be published, the same holds true for the bactericidal substances.

Against this view Buchner, while in general he confirms our findings of fact, maintains that the thermostable substances of normal sera are not analogous to the immune bodies, but are something apart by themselves. He therefore gives them a distinct name, "Hilfskörper" [=aiding body]. Such a separation of the connection between the physiological and the pathological is opposed to the teachings of Virchow. Aside from this, however, I regard the proof which Buchner advances for placing these "Hilfskörper" by themselves as insufficient. It is entirely negative and consists in this, that, according to Buchner, proof has not been offered that in normal haemolysis a "Hilfskörper" does not always come into action. Against this I should like to point out that, in the very large number of cases of normal haemolysis studied during the past years by myself and fellow workers, we have always succeeded in discovering the amboreceptor effecting the action. At times, of course, this required a great deal of labor and trying all sorts of sources for complement. Experiments like those recently published by Buchner, in which only one combination chosen at random from the many possible ones is employed, do not argue against the presence of amboreceptors in case the experiment results negatively, for no one versed in this subject would assume that every amboreceptor must find a fitting complement in every serum used. Hence Buchner does not furnish any proof that haemolysis can be produced by the alexin alone.

In connection with this I should like to call attention to the fact that the alexin or complement action possessed by normal serum is due to a plurality of substances, not to a single one. Each complement by itself is harmless, for only through the intervention of the amboreceptor is its injurious action carried over to certain tissues. When this occurs, however, the action is the same on its own as on foreign tissues. It is surprising to watch how guinea pig blood-cells which have been loaded or sensitized with certain amboreceptors at once dissolve if their own serum is added, this serum now acting as a deadly poison. There is very little ground, therefore, to regard the complements as playing the rôle of defenders against foreign invaders.

That they appear to play this rôle is due to the action of what I have termed the "horror autotoxicus," which prevents the production within the organism of amboceptors directed against its own tissues.

In this "horror autotoxicus" we are dealing with a well-adapted regulatory contrivance which it may be well to discuss briefly. The investigations of numerous authors have shown that by injecting animals with any kind of foreign cell material cytotoxic substances can be produced directed exactly against the material used for immunization. Thus if a dog is immunized with an emulsion of goose brain, it will be found that the dog's serum will be highly toxic only for geese, killing these animals with cerebral symptoms. In the same way we can produce other poisons, hepatotoxins, nephrotoxins, etc., each of which acts only on a certain organ of a particular species. In human pathology, however, we must consider the absorption of the body's own constituents and not of those of other bodies. The former may occur under many conditions; for example, in hæmorrhages into the body cavities, in the absorption of lymph-gland tumors, in the febrile waste of body parenchyma. It would be dysteleological to the highest degree if under these circumstances poisons against the body's own parenchyma, autotoxins, were to arise. I have attempted to solve this question by injecting goats with the blood of other goats. The sera of animals so treated did not dissolve their own blood-cells, but dissolved those of other goats. Hence it did not contain an autotoxin, but an "isotoxin," in conformity with the law to which I give the name "horror autotoxicus."

I believe that the isotoxins may perhaps come to play an important rôle in diagnosis and pathology. In the serum of dogs in which he had produced a chromium nephritis, Metchnikoff found that an isonephrotoxin had developed, for when this serum was injected into normal dogs it produced a nephritis. It is more than probable that in man also the greatest variety of isotoxins is formed. In the case of the blood this has already been positively demonstrated by a number of authors, such as Landsteiner, Ascoli, etc.

With the exception of the red blood corpuscles we cannot, of course, undertake any studies in man concerning the isotoxins of the parenchyma. Many considerations, however, indicate that it will be possible to carry out these experiments on monkeys and so gain a new foundation for pathology and therapy in man.

The number of combinations present in the blood serum and making up the ever-changing haptin apparatus is infinitely great.

Of these especially the substances of the amboceptor type are in most intimate relationship to the processes of natural immunity, for it is they which, in conjunction with the complement, effect the destruction of the injurious bacteria. Hence if there is a loss of natural immunity, it will next be necessary to inquire whether there is a lack of complement or of amboceptor.

I am convinced that these haptin studies open up a new and important field of biological investigation and will add to our knowledge concerning the process of assimilation. Clinically they should be of even greater importance. Since I am not in the position to make such chemical investigations on an abundance of material, I have thought it my duty to clearly define my point of view, thus furnishing to others the basis for a proper study of this subject. The significance of this method for pathology and therapy will not perhaps be fully realized until after the lapse of years.

### XXXIII. THE RECEPTOR APPARATUS OF THE RED BLOOD-CELLS.<sup>1</sup>

By Professor Dr. P. EMMICH.

We know of a large number of agents which are able to injure the red blood-cells or kill them. In a study entitled "Zur Physiologie und Pathologie der rothen Blutscheiben" (Charité Annalen, Vol. 10) I have shown that solution of red blood-cells is brought about by all agencies (mechanical, chemical, or thermal) which kill protoplasm. At that time I had already expressed the hypothesis that the erythrocytes possessed a peculiar protoplasm, the discoplasm, whose chief function consists in preventing the escape of the haemoglobin into the blood plasma. If the discoplasm is killed, the haemoglobin will immediately diffuse, i.e., the blood becomes laky. This process is in no way connected with conditions of osmotic tension, for in many blood poisons, such as digitoxin, veratrin, solution corrosive sublimate, etc., this destruction takes place in very high dilutions which hardly change the molecular concentration at all.

The ordinary blood poisons, and they are very numerous (arsenium bodies, helvellie acid, aldehydes, polyphenols, etc.), are chemically clearly defined substances; they exert their deleterious action in exact accordance with the principles which we have already studied in connection with the distribution of pharmacological substances, such as alkaloids, etc. Recently, however, we have come to know another group of blood poisons which exert their injurious action after the manner of toxins, i.e., through the agency of special haptophore groups which fit into suitable receptors. All of these substances are highly complex derivatives of living animal or vegetable

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<sup>1</sup> Reprint from: Schlussbetrachtungen, Erkrankungen des Blutes. Nothnagel's Spezielle Pathologie und Therapie, Vol. VIII, Vienna, 1901.



cells; for the present at least their chemical nature is unknown. Into this class, to mention only the simplest types, belong the following:

1. *Poisonous phytalbumoses*; ricin, abrin, crotin, phallin;
2. *Bacterial secretions*, tetanolysin (Ehrlich, Madsen), staphylo-toxin (van de Velde, M. Neisser, and F. Wechsberg), pyocyanous poison (Bulloch), streptococcus poison (v. Lingelsheim), cholera poison, and probably many others.
3. *Poisonous animal secretions*, especially the various snake venoms.

The majority of these substances, especially all of the bacterial products, produce ordinary hemolysis. In contrast to this, as Robert has shown, abrin and ricin cause a rapid clumping of the erythrocytes, a process which is analogous to the agglutinative phenomena studied by Gruber, Durham, and Widal. However, in the case of the poisonous phytalbumoses we cannot assume that there is an essential difference between hemolysis and agglutination, because one of them, crotin, has been shown by Elfstrand to exert a pure-agglutinating action on certain species of blood (sheep, pig, ox) and a pure solvent action on others (rabbit).<sup>1</sup>

Of especial importance, however, is the fact that all these poisons on being introduced into the animal body produce specific antitoxins (antiricin, antiabrin (Ehrlich); anticrocin (Morgenroth); anti-tetanolysin (Madsen); antileucocidin (van de Velde). In view of what we have already discussed this fact alone is sufficient to ascribe to these substances the possession of a haptophore group through which they exert their toxicity. Furthermore, just like the true toxins, they possess a second group which is the cause of the toxic action. As Madsen has shown in the case of tetanolysin, and M. Neisser and F. Wechsberg for staphylolysin, it is possible to change these poisons into modifications which have more or less completely lost their toxicity but which preserve unchanged the properties dependent on the possession of the haptophore group (allinity for the antibody, production of immunity). These modifications, first recognized

<sup>1</sup> Even ricin, which is apparently purely agglutinating, exerts an action on the discoplasm which causes hemolysis. In the ordinary technique of the experiment this action is obscured by the fact that in the agglutinated masses the conditions are very unfavorable for diffusion. If these conditions are made more favorable by breaking up the clumps by shaking one can easily observe the escape of the hemoglobin.

by me in diphtheria poisons, depend on the separate destruction of the very unstable toxophore group.

In passing now to the substances contained in blood plasma I shall discuss first the agglutinins. Even normal serum frequently contains substances which clump certain bacteria and erythrocytes. Although at first, in accordance with Buchner's views, one single substance was made responsible for the different actions I believe that at present the pluralistic standpoint first maintained by me is generally accepted. The plurality of normal agglutinins was at once proven as soon as my principle of specific combination was applied to this question, as was done by Bordet and Mallow. The latter showed that if goat serum which agglutinates the erythrocytes of pigeon, man, and rabbit is shaken with the red cells of one of these species, e.g. pigeon, it will be found that the centrifuged fluid still contains the two other agglutinins unchanged, whereas the agglutinin for pigeon blood is absent.

These substances can be obtained artificially by following the procedure of Belfanti and Carbone, who injected animals with considerable amounts of foreign red blood-cells (blood-cell immunization). They are readily separated from the hemolysins developing simultaneously by heating for half an hour to 56° C. As a result of this the action of the ambceptor lysins is destroyed while the agglutinins themselves are unaffected. To be sure if the temperature is increased to 70° C. it is possible to destroy also the agglutinating action. In that case, however, the addition of normal serum no longer exerts a reactivating action. From this it follows that the agglutinins<sup>1</sup> are not of such complex constitution as the ambceptor lysins, analogous to the toxins they contain a haptophore group and a zymophore which causes the coagulation process. In accordance with this I believe that the agglutinins are nothing more than *receptors of the second order*.<sup>2</sup>

<sup>1</sup> The agglutinins here described, in contrast to yam and abrin, give rise to no further injurious action on the discoplasm.

<sup>2</sup> In the first part of "Schlussbetrachtungen" I have distinguished

1. *Receptors of the first order*, which concern themselves with the assimilation of simple substances (toxins, ferments, and other cell secretions). For this purpose a single haptophore group suffices. When thrust off into the blood in consequence of the introduction of toxins, these receptors constitute the antitoxins (antiferments).

2. *Receptors of the second order*, which in addition to the haptophore group possess a second group which effects the coagulation. After they have been

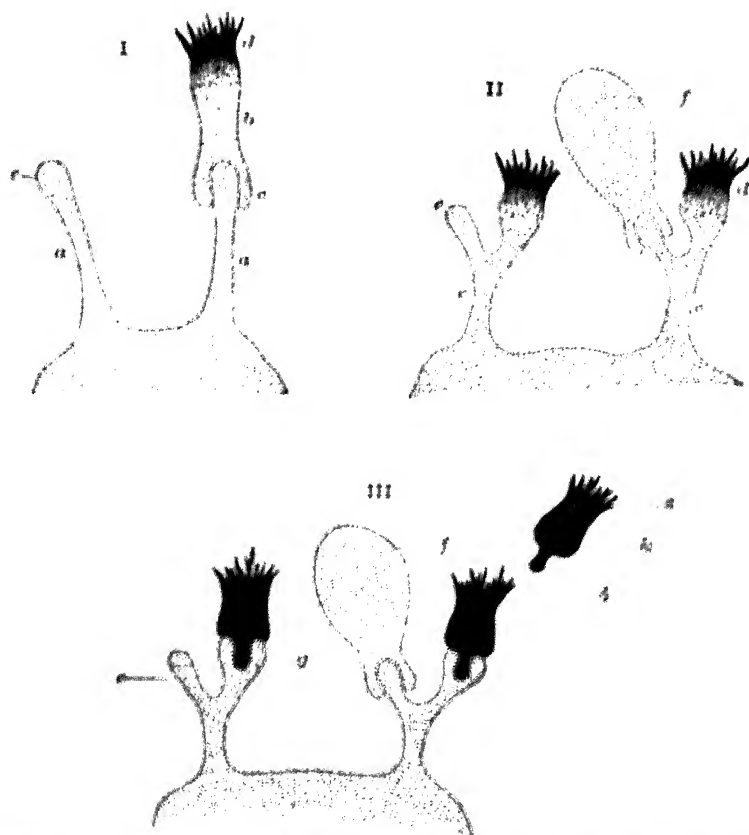


FIG. 1.—THE VARIOUS TYPES OF RECEPTORS ACCORDING TO EHRICH.

- I. *Receptors of the First Order*.—This type is pictured in I. The portion *a* represents the haptophore group, whilst *b* represents a toxin molecule, which possesses a haptophore group *c* and a toxophore group *d*. This represents the union of toxin and antibody, or ferment and antid ferment, the union between antibody and the toxin or ferment being direct.
- II. *Receptors of the Second Order* are pictured in II. Here *a* represents the haptophore group, and *d* the toxophore group of the receptor, *f* being the food molecule with which this receptor combines. Such receptors are possessed by agglutinins and precipitins. It is to be noted that the toxophore group is an integral part of the receptor.
- III. *Receptors of the Third Order* are pictured in III, *a* being the haptophore group and *g* the complementophile group of the receptor. The complement *k* possesses a haptophore group *k* and toxophore group *i*, whilst *f* represents the food molecule which has become linked to the receptor. Such receptors are found in hemolysis, bacteriolysis, and other cytolytic unions with these cellular elements being effected by the antireceptor (a throat off receptor of this order). It is to be noted that the digesting body, the complement, is distinct from the receptor, a point in which these receptors therefore differ from those of the preceding order.

Next we come to the very important substances in serum which cause hæmolysis. I have previously dwelt in detail on the fact that in this the action is always due to amboceptors which attract both blood-cells and complement. Hence I may limit myself at this time to some supplementary remarks. It has long been known that the blood serum of one species injures and dissolves the erythrocytes of other animal species. This is the case not only in distantly related types, such as fish and mammals, but, as was shown by therapeutic blood transfusions, occurs also in comparatively near relatives. Buchner was the first to appreciate the significance of this phenomenon, and assumed that the serum contained a substance innocuous for its own body but acting destructively on foreign elements (bacteria and blood-cells). This substance he therefore terms alexin. Not until, in later years, the mechanism of artificially produced lysins became clear was this unitarian view shown to be untenable. First it was found that the lysins contained in normal blood are not simple in nature, but are composed just like those artificially produced, of two components, the amboceptor and the fitting complement. Furthermore, corresponding to the results in the case of agglutinins, and by means of the same methods, it was found that a given serum can contain a large number of different amboceptor lysins. If a certain serum (e.g. dog serum) dissolves the erythrocytes of different species, the specific combining method has shown that this property is due to the presence of different amboceptors, each of which is related to only one of these species of blood-cells. In fact it even seems as if different complements may correspond to these amboceptors.

In view of what has been said we are fortunately able to regard these different agents which injure the blood from a common point of view. Whether we are dealing with vegetable or animal products, whether with lysins or agglutinins, whether with substances of toxin-like nature or of the complex amboceptor type,—*in all of these cases the prerequisite and cause of this poisonous action is the*

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thrust off into the blood they constitute agglutinins and precipitins. The toxins also are to be regarded as receptors of the second order thrust off by bacteria.

3. *Receptors of the third order*, which possess two haptophore groups, one of which effects the union with the foodstuff, whereas the other lays hold on certain substances circulating in the blood plasma, the complements, which cause ferment-like actions. After they are thrust off these receptors constitute the "amboceptors."

*same, namely, the presence of suitable receptors on the blood-discs, i.e., receptors which fit the haptophore groups of the toxin or the corresponding groups of the amboceptor.* This view, already generally accepted for the toxin poisonings, is supported by considerations of two kinds. First is the positive proof in the case of the manifold blood poisons, that their injurious action is always preceded by the anchoring of the poison to the blood-cell. Only such species of blood-cells are susceptible to a certain hæmolsin which are able to anchor the same. This has been confirmed again and again in the case of amboceptor lysins. Conversely, therefore, there is the closest connection between natural immunity and absence of receptors. That the fixation of the poisons is not due to mechanical effects, such as surface attraction, but to a true chemical process, is at once shown by the strict specificity which obtains. This is observed especially in the amboceptor lysins produced artificially. This specificity is in marked contrast to the many-sided and non-selective action of surface attraction (charcoal, etc.). The second point which supports the above view is the fact that the action of a certain poison, and only of this one, is inhibited by the corresponding antitoxin. According to my views, the action of antitoxins is explained by assuming that they occupy the haptophore groups of the toxin molecule and so prevent these from combining with the receptors of the tissues. It is quite incomprehensible to me how the specificity of the antitoxins can more easily be explained on the basis of the mechanical conception.

This brings us to a very important point, namely, the surprising plurality of receptors. Even in the blood poisons each antiserum protects only against the substance through which it was produced by immunization. This law of specificity, which has so repeatedly been confirmed in the infectious diseases, is thus seen to apply here without any change. Antiricin serum protects the blood-cells only against ricin, antitetanolysin only against tetanolysin, every anti-amboceptor only against a corresponding amboceptor.

Hence in every species of blood-cell we shall have to assume the existence of as many different kinds of receptors as there are poisons. This is obviously a very large number. Thus if the blood-cells of rabbits are injured by ricin, croton, abrin, phallin, by the most diverse products of bacterial metabolism, and by a large number of sera of other species, we shall have to assume a certain receptor (ricin receptor, etc.) for each case. Almost every day, however,

we are coming to know more such blood poisons; the number of different receptors which we can determine, therefore, continues to increase.

In this connection I should like to present the results which Dr. Morgenroth and I have obtained in attempting to produce autolysins by immunizing goats with blood from the same species instead of blood from foreign species. In only one single instance were we successful, i.e., in obtaining a solution of the animal's own blood-cells. In all other cases we obtained merely an isolysin, which dissolved the blood-cells of other goats but not those of the goat immunized. If the blood of a large number of goats is tested with a particular isolysin, it would be found that of some goats the blood is highly susceptible, of others it is feebly susceptible, and of still others the blood is not at all susceptible. In the case of the susceptible bloods it can be shown that the isolysin consists of the amboceptor which is anchored, plus a complement of normal goat serum. In course of time we have produced thirteen such isolytic sera, and found to our surprise that they all differed from one another, i.e., that they represented different isolysins. Thus the first serum dissolved the blood-cells of A and B; a second serum those of C and D; a third A and D, etc. By means of this one experiment we have, therefore, come to know thirteen different lysins, to which, of course, a similar number of receptors must correspond. It was fortunate for us that in the blood-cells of an animal all the receptors were not present, but only a part of the same, for it was only owing to this fact that a separation of the different kinds was possible.

It is worthy of note that many receptors may be present in the blood-cells in relatively large amounts. If we designate as the single lethal dose (L.D.) that amount of a certain amboceptor which when supplied with sufficient complement just suffices to completely dissolve a constant amount of blood, we can, by employing different amounts of amboceptor solutions inactivated by heat, readily determine how many L.D. can be anchored by the amount of blood in question. As a result of this it has been found that in some cases only just the single L.D. is bound. More frequently the combining power of the erythrocytes is much higher, so that two to ten and even fifty times the L.D. is bound. In such cases, therefore, we are dealing with a marked excess of these particular receptors. An analogous case, by the way, has long been known as a result of Wassermann's experiment concerning the power of brain substance

to bind tetanus poison. In virtue of such an excess of tetanus receptors, the brain also absorbs a considerable multiple of the L.D. Hence in test-tube experiments it is still possible to neutralize considerable quantities of poison with the brain of a guinea-pig which has died of tetanus.

All of these facts lead to the conception that the red blood-cells possess an enormous number of receptors which probably belong to hundreds of different types. Of these, again, a few may be present in relatively large quantities. This fact is surprising; for in a way it is opposed to the view held until now concerning the function of the red blood-cells. It is inconceivable that the simple interchange of oxygen, a purely chemical function of the hæmoglobin, would require so complex an arrangement as that just described. In my opinion, therefore, this enormous apparatus indicates that the red blood-cells actually exercise properties which we have thus far overlooked. If we consider that the receptors in general serve to take up foodstuffs, or in some cases the products of internal metabolism, we may easily assume that the receptor apparatus of the erythrocytes fulfills this same purpose. Since, however, we know that the *vita propria* of the blood-cells is very limited, we shall have to assume that the substances taken up are not for the blood-cells' own consumption, but are designed to be given off to other organs. The red blood-cells may therefore be regarded as storage reservoirs in the sense that they temporarily take up the most varied substances derived from the food or from the internal metabolism, provided these substances are supplied with haptophore groups. I may be permitted to call attention to the fact that the erythrocytes contain chiefly receptors of the first order,<sup>1</sup> i.e., receptors which take up substances but do not further digest them.

After these explanations I feel justified in believing that the study of receptors has opened up a new and important field of biological investigation. In order to make my meaning clearer I should like to quote the following paragraph from Verworn (*Beiträge zur Physiologie des central Nerven-Systems*, I. Thiel, page 68) in which our present knowledge is reviewed: "The living substance of every cell, so long as it actually is living and manifests vital phenomena, is constantly decomposing automatically and constantly forming new substances. Dissimilation and assimilation are the fundamental

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<sup>1</sup> See note, page 392.

phenomena of metabolism, while they are also at the same time the two phases of the vital process.

"As a result of a large number of facts we have, as is well known, arrived at the conclusion, confirmed chiefly by Pflüger, that the end-point of metabolism is represented by complicated combinations of egg albumin called by Pflüger living albumin. Such combinations are exceedingly labile, decomposing to a certain extent spontaneously, and to a greater degree in response to stimuli. In these combinations we are dealing with chemical substances whose molecules, just because of this easy decomposition, disclose a chemical constitution quite different from the lifeless albuminous bodies which we know. I have therefore proposed to replace the name 'living albumin molecule' by the term 'biogen molecule'. *The decomposition and production of the biogens is therefore the cornerstone of the vital process in every living cell.* The substances given off by the cell are derived from the decomposition of the biogens, the material for the formation of new biogen molecules is furnished by the food taken up and transformed by the cell. I have, however, called attention to the fact that this view needs to be extended in one direction (Allg. Physiologie, Jena, 1897). A number of facts indicate that the decomposition of the biogen molecule is not complete and that all of the atomic groups thus arising are not given off by the cell."

In view of these explanations Verworn assumes that in the decomposition of the biogens a residue is always left which again takes up food substances and so regenerates the biogen molecule. It seems to have entirely escaped Verworn that I had expressed entirely analogous views in much greater detail twelve years previously ("Über den Sauerstoffbedarf des Organismus," Berlin, 1885). I assumed that the specific function of the cell is dependent on a central group in the living protoplasm, of peculiar structure; furthermore, that atoms and atomic groups are attached to this central group as side-chains. *These side-chains are of subordinate importance for the specific cell function, but not so for the life itself.* I also said that everything indicated that it was just through these indifferent side-chains that physiological combustion was effected, for one portion of these side-chains effects combustion by giving off oxygen, the other portion being thus consumed. On page 11 of this monograph I expressed myself as follows: "The question as to the manner in which the side-chains constantly being consumed



are regenerated must, of course, excite the greatest interest. It can be conceived that certain portions of the functional central group [Leistungskern] can fix combustible molecular groups, and that these groups are thus rendered more susceptible to complete combustion."

It is at once clear that these fixing portions which I now term receptors, correspond exactly in their nature to the loosen residues of Verworn.

Probably no one who has seriously studied these questions will question the importance of these deductions. In spite, however, of the decades which have elapsed since Pflüger's publication we have not advanced one step in our experimental knowledge of this subject, a fact which is due to the endless difficulties occasioned by the nature and instability of the living material. I hope that my theory is destined finally to bridge this wide gape. The knowledge that the numerous antibodies are nothing more than thrust off receptors of the cell should make it possible to get at the nature of assimilating processes. By means of immunization we can compel the thrustings off of certain particular receptors which then collect in the serum. Free from the disturbing connection with the protoplasm, they no longer offer any difficulties for biochemical investigations. Viewed in this light, I believe that the facts which I have determined concerning the action of unireceptors and ambireceptors constitute a new step toward a true conception of the vital processes.

It can hardly be doubted that the red blood-cells, owing to their relatively simple structure and the ease with which they can be manipulated, are better adapted for these purposes than other cellular elements. I also believe that clinical investigations are destined to play a leading rôle in the solution of these problems, simply because the various types of disease offer a much greater variation in the vital conditions than we can attain by means of experiments. Even aside from the gain to pure biological science, clinical medicine should derive the greatest advantage from such studies, for, as already mentioned, they deal with the true conception of the pathology of the red blood-cells.

In order somewhat to facilitate such a study it may perhaps be well to give a brief sketch of the facts which in conjunction with my colleague, Dr. Morgenroth, I have discovered regarding the physiology of the receptors.

Considering the large number of receptors which each species

of blood cell possesses, it is not surprising that certain types are common to the majority if not to all the vertebrate species. In this connection I shall only point out the fact that receptors for ricin, abrin, ichthyotoxin (which injure a large number of different erythrocytes) are widely distributed in the animal kingdom. Side by side with such generally distributed groups, however, there are types which are limited to a comparatively small group of animal species. Thus by means of cross immunization we have demonstrated that the blood-cells of goat and sheep possess several special receptors in common. This was shown by the fact that the isolysins obtained by injecting goats with goat blood usually effected solution of sheep blood-cells, although to a less degree. In making the counter experiments, immunizing goats with sheep blood-cells, we obtained in addition to sheep lysin the isolysin acting on goats.

Besides this there are groups of receptors which are specific for each animal species. This is best shown by the normal course of the Belfanti-Bordet experiments. In these as a rule only specific hemolysins are formed, i.e., hemolysins directed against the erythrocytes exciting the immunization<sup>1</sup>

Such variations in the zoological distribution of certain receptors (also of the complements, etc.) is readily explained by the very natural assumption that the metabolic processes, whose indicator the receptors really are, show corresponding variations. It is just as little to be doubted that certain assimilative processes are specific for only one species of animal as that others occur in exactly the same manner in man and in the frog.

It is also of considerable importance that in any given animal species a considerable individual variation of the receptors may occur, a fact first observed in experiments with crotin on rabbits. The strongest confirmation of this point is the result of our experiments on goat isolysins. As already stated, out of the goats we used there were always only a few which reacted to one of the thirteen different isolysins.

Through the opportunity so offered we convinced ourselves of another important fact, namely, that the susceptibility of a given individual can change in a comparatively short time. We found that a goat which reacted to a certain isolysin became unsusp-

<sup>1</sup> We have obtained entirely analogous results also with other constituents of blood serum, e.g., with complements

tible after several weeks, and further that in this case there had been a disappearance of the special receptors previously demonstrated as present. We have also encountered the reverse of this, namely, the appearance of receptors previously absent.

Evidently this coming and going of certain receptors reflects internal metabolic processes which may be dependent on a large number of external or internal factors. In this connection a fact observed by Kossel is especially interesting. This observer found that during the course of immunization with eel blood the blood-cells of rabbits acquire a high degree of resistance against the poison, a fact which we should perhaps ascribe to a lack of receptors. In this case we are dealing with something which is specific for the immunization with eel blood; for we could not obtain these results with two other blood poisons, croton and tetanolysin.

To a certain extent the experiments of Kossel, Gley, and Tschistowitsch furnish a clue to the mechanism of these phenomena. They show that the first phase of immunization is that of antitoxin formation, and that the unsusceptibility of the red blood-cells is not developed until later.

The way in which blood-cells which have previously been susceptible to a certain poison become unsusceptible to this can very readily be explained. We have seen that those blood-cells, which are susceptible to the action of a poison (e.g., eel blood) possess appropriate receptors. Under physiological conditions the office of these is to anchor a certain particular product of metabolism,  $x$ . If now through treatment with the poison the specific antitoxin is produced, it is clear that this antitoxin when present in the circulation is able to anchor not only the poison but also the normal metabolic product,  $x$ , thus preventing the latter from combining with the erythrocytes. Since this, however, renders the corresponding receptors permanently useless, the possibility of their disappearance is at once given—after the manner of atrophy through disuse. This will occur most readily in those cases in which the substance  $x$  can readily be spared by the cell, i.e., cases in which (as in sugar) the substance can be replaced by some other kind of material (e.g., fat).

A disappearance of the receptors can, however, occur without the development of such a deflecting antibody, as is shown by the isolysin experiments. The most natural conclusion is that the lack of receptors in this case is produced by an inconstant, perhaps only

a temporary, metabolic product. Perhaps this can be brought into connection with the interesting observation of Gilex that the blood-cells of new-born rabbits are highly resistant against red poison, acquiring the normal high susceptibility only in the course of weeks.

Be this as it may, everything indicates that there is an organic harmonious connection between the metabolism of any given period and the nature of the receptors present. This connection depends on the fact that substances with haptophore groups exert a stimulus on the protoplasm which excites the production of the receptors in question.

In conclusion I wish to point out that many facts indicate that the species of receptors found in the erythrocytes may also be present in the cells of other organs. Thus, mentioning only one example, tetanolyisin is anchored not only by the erythrocytes, but also by the brain and other organs. This phenomenon also shows itself in the immunizing test. Von Dungern, for example, found that serum of rabbits which had been treated with tracheal epithelium of oxen exerted a marked hemolytic action on ox blood in addition to its injurious action on epithelium. Metchnikoff's objection that this was due to an error in technique (the injection of admixed blood-cells) was controverted by von Dungern, who showed that injections of cow milk, a material absolutely free from blood-cells, produced the same hemolysins. It follows that certain receptors must be common to the red blood-cells and the epithelial tissue or the milk derived from this.

The wide distribution of a particular combining group harmonizes very well with the assumption discussed above concerning the functions of the receptor apparatus of the red blood-cells.

According to Miescher's comparison the red blood-cells serve as a sort of bank of deposit where the metabolic products in excess at any given time may be stored temporarily. In this case the substances will be yielded up only to organs possessing suitable receptors. This process will be all the more complete if the affinity of the tissue receptors is greater than that of the blood receptors. There are many reasons for believing that the affinity of the tissue receptors is not constant, and that it can be considerably increased through certain stimuli (assimilative stimuli). It is obvious that hunger, if we may apply the term to purely cellular processes, must constitute one of the most important assimilative stimuli. This functional in-

crease of affinity would constitute a wonderful illustration of how well the process of assimilation is adapted to its purpose.

NOTE.—Subsequent addition to page 490:

Calmette also has recently reported (*Compt. rend. de l'Académie des sciences*, T. 134, No. 24, 1902) that the blood cells of animals highly immunized with cobra poison preserve their sensitiveness completely against the lysis by the cobra poison. In a goat highly immunized with ricin Jacoby (*Beiträge z. chem. Physiologie und Pathologie*, Bd. II, 1902) was unable to discover any increased resistance of the red blood cells against the action of the ricin.

## XXXIV. THE RELATIONS EXISTING BETWEEN CHEMICAL CONSTITUTION, DISTRIBUTION, AND PHARMACOLOGICAL ACTION.<sup>1</sup>

(An Address delivered in the "Verein für innere Medicin," Dec. 12, 1898.)

By Professor Dr. P. Langer.

UNTIL recent years the relations between chemistry and medicine were in general confined to purely scientific questions. In the last decade, however, a change has taken place, such as has rarely been seen in the history of medicine. One is justified in saying that at the present time the chemical view constitutes the axis about which the most important views in medicine turn, and that the two poles are the synthetic construction of new therapeutic agents on the one hand, and the discovery of specific therapeutic products of living cells on the other. The contrast between these two methods is very pronounced. In the first case, one makes use of the retort and simple, definite reactions; in the other, of the mysterious powers of living nature so infinitely well suited to their purpose. A greater contrast cannot be imagined than that existing between the modern medicaments, whose constitution is known down to the finest details, and diphtheria antitoxin, which we know only through its specific action and about whose chemical constitution we know absolutely nothing. Thus far the genius of the most eminent chemists has not availed to produce these bodies in a pure form and get an insight into their chemical nature. All that this endless study has brought forth is the conviction that we are dealing with atomic groups of the utmost complexity, which for the present are entirely beyond our chemical researches and which, so far as we can see, will long remain so.

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<sup>1</sup> Reprint from the v. Leyden Festschrift, Vol. I.

As a result of this and other considerations the view has become prevalent that the chemo-therapeutic and the bio-therapeutic tendencies are absolutely different from each other. As late as two years ago a certain high authority said that the antitoxins act after the manner of specific forces (in a physical sense). If this theory of "forces" were to be upheld every possibility of bridging the contradictions would be completely lost, for then every tertium comparationis would be lacking.

If instead of this we assume that both kinds of substances exert their power by purely chemical means, we shall find that certain questions arise which are of great significance for the further development of therapeutics. Convinced that this is correct I have busied myself during the past ten years with attempts to prove the chemical theory of toxins and antitoxins experimentally. I believe I am justified in claiming that I have caused the chemical conception to be accepted among ever-widening circles. This I have accomplished:

1. By the introduction of the test tube experiments.
2. By systematic investigations concerning the mutual neutralizing affinities.
3. By the demonstration of toxoids and their various modifications.

### I.

If then the medicaments of known constitution and the biotherapeutic products, both act only in a chemical manner, i.e., if both effect the organism chemically, the first problem to be solved is to determine on what factor the very dissimilar action of these two classes of bodies depends. It will be well to begin with the simplest condition, and to study first the mode of action of bodies whose chemical constitution is well known.

It is particularly desirable to gain an insight into the relations existing between chemical constitution and pharmacological action. During the last few decades these have come to play an important rôle in the modern synthetic tendencies. The history of this tendency is comparatively recent, dating from the year 1859 when Stahlschmidt demonstrated that strychnine loses its tetanizing action when a methyl group is introduced, being transformed into a curare-like poison. In view of the fact that this methylation forms an ammonium base, Fraser and Braun studied a number of other ammonium bases derived from various alkaloids and found that all of these bodies



possessed a curare-like action. Since that time a large number of ammonium bases derived from the most varied alkaloids have been investigated, most all of which showed the same action. The final step was achieved only recently when Bodin showed that curarin is itself an ammonium base. He found that the curares contain a tertiary alkaloid, curin, which is of slight toxicity. If this curin was subjected to methylation an ammonium base was formed which corresponded completely in properties and actions with the natural curarin, but was about 260 times as toxic as the original substance. Since this time these questions have been studied on many different combinations by a large number of investigators, among whom may be mentioned Nencki, Jaffé, Fildine, Mering, Branton, Brugger, Gildes, and Aronson. I cannot, however, go into details and must confine myself to giving a short epitome of what has been done in the development of synthetic remedies.

First in importance are the artificial antipyretics, of which the main types are the antipyrin series and the phenacetin series. The history of the origin of these two groups is absolutely unlike. In one case the starting-point was the fact that quinine contains a hydrated chinolin derivative, by means of simpler combinations it was attempted to obtain the same end. Finally, after chinolin, kairin and thallin had proved of such little value, antipyrin was obtained and found most useful. The second group, which includes phenacetin and its numerous relatives, owes its discovery not to theoretical speculations but to a coincidence, the result of an error.

Of the other therapeutic agents the discovery of the hypnotic action of sulfonal by Baumann has proven of great practical and theoretical significance. The same holds true of the production of the new anesthetics (orthoform and curamin), which was closely connected with the discovery of the constitution of curamine. In recent years efforts are constantly being made to do away with the by-effects possessed by certain remedies, such as guaiacol and formaldehyd. These efforts, first undertaken by Nencki, seek by means of suitable combinations and cleavages to give rise to a gradual liberation of the active component. While of great practical value they have but little interest in the question concerning the connection between constitution and action.

When now we come to inquire what conclusions we can draw from the study of the large number of therapeutic agents, which now embrace many hundreds of different remedies, conclusions which



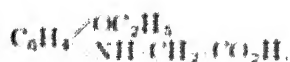
will apply to the study of the relation between constitution and action, we find that the results are still very meagre.

In the main they are as follows:

1. The discovery that the antipyretic action of the anilin and amidophenol derivatives (phenacetin) is proportional, within certain limits, to the amount of p-amidophenol split off in the organism (Hinsberg). Hence all such combinations in which, through improper substitution of the amide group or of the main group (p-amidoacetophenon,  $\text{NH}_2\text{-C}_6\text{H}_4\text{-CO-CH}_3$ ), the liberation of p-amidophenol is prevented cannot be used as antipyretics.

2. The discovery by Kendrick, Dewar, Filchne, that in the pyridin series the hydrated products act more strongly than the parent substance. Thus piperidin,  $\text{C}_5\text{H}_{10}\text{NH}$ , is a much stronger poison than pyridin,  $\text{C}_5\text{H}_5\text{N}$ . In this the transformation of the tertiary nitrogen atom in the imin group plays a certain rôle, as is shown especially by the observations of Filchne on the tetra hydro chinolin series. According to these the replacement of the methyl hydrogen atom by alcohol radicals reduces the irritant action.

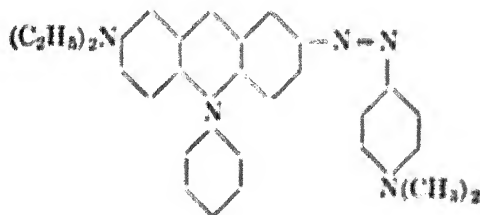
3. The demonstration that the antipyretic power of antipyretics is destroyed by the introduction of salt forming acid radicals, such as  $\text{SO}_3\text{H}$ ,  $\text{CO}_2\text{H}$  (Ehrlich, Aronson, Nencki, Benzoldt). Hence so far as this action is concerned acetamidoacetic acid,  $\text{C}_6\text{H}_5\text{N}(\text{COCH}_3)\text{CH}_2\text{CO}_2\text{H}$ , is inert. So also are acetanilid sulfonic acid,  $\text{C}_6\text{H}_5\text{-NH-CO-CH}_3\text{SO}_3\text{H}$ , the carbonic and sulfonic acids of phenacetin, and the ethoxy-phenylglycin which is similar to phenacetin.



4. The demonstration by Filchne, Einhorn, Ehrlich, and Poulson, of the anesthesiophore character of the benzoyl radical. Homologues of cocaine, such as are obtained when other acid radicals, such as succinic acid, phenylacetic acid, cinnamic acid, are introduced into the regoninmethylester, lack these anesthetic properties. This discovery resulted in the production of new potent anesthetics containing the benzoyl group as the active agent, e.g. eucain (Merling) and orthoform and nirvanin (Einhorn).

5. The function of the ethyl group. This has been brought out very clearly by Baumann's discovery that the hypnotic action of certain disulfons is due exclusively to the presence of ethyl groups

and that it increases with the number of these groups: thus sulfonal,  $(\text{CH}_3)_2\text{C} \cdot (\text{SO}_2\text{C}_2\text{H}_5)_2$ , and trional,  $\text{CH}_3\text{C}_2\text{H}_4 \cdot \text{C} \cdot (\text{SO}_2\text{C}_2\text{H}_5)_2$ . Of the other hypnotics which owe their action in part to the ethyl group I may mention amylenhydrate,  $\text{C}(\text{CH}_3)_2(\text{C}_2\text{H}_5)_2\text{OH}$ , and ethyl urethan,  $\text{NH}_2 \cdot \text{CO} \cdot \text{OC}_2\text{H}_5$ . The influence of the ethyl radical is furthermore clearly shown in another series of combinations. In an artificial sweetening substance, dulcin, which is about two hundred times sweeter than sugar, this influence is very evident. This substance is phenyl urea ethoxylated in the para position,  $\text{C}_2\text{H}_5\text{O} \cdot \text{C}_6\text{H}_4 \cdot \text{NH} \cdot \text{CO} \cdot \text{NH}_2$ . Since neither simple phenyl urea nor the methoxy condensation,  $\text{CH}_3 \cdot \text{O} \cdot \text{C}_6\text{H}_4 \cdot \text{NH} \cdot \text{CO} \cdot \text{NH}_2$  analogous to dulcin, possesses any sweet taste whatsoever, we are forced to conclude that this is due to a function of the ethyl radical. Of the remedies containing the ethyl radical there may still be mentioned phenacetin,  $\text{C}_2\text{H}_5\text{O} \cdot \text{C}_6\text{H}_4 \cdot \text{NH} \cdot \text{CO} \cdot \text{CH}_3$ , and two anesthetics, holocain,  $\text{C}_2\text{H}_5\text{O} \cdot \text{C}_6\text{H}_4 \cdot \text{NH} \cdot \text{C}(\text{CH}_3)_2 \cdot \text{N} \cdot \text{C}_6\text{H}_4 \cdot \text{OC}_2\text{H}_5$ , and acain, all three of which are derived from phenetidin. It is significant that of the entire series of alcohols only ethyl alcohol has become established as a beverage, and that since the earliest time attention was directed to producing it as pure as possible, i.e., to free it from higher and lower relatives. In all of these examples we are dealing with an influence on the nervous system, the central system (sulfonal ethylurethan, amylen hydrate, alcohol), as well as the peripheral endings (dulcin, anesthetics). Hence we shall probably not err if we assume that the ethyl group possesses a certain relation to the nervous system. In this connection an observation which I made in conjunction with Dr. Michaelis may perhaps be of some significance. We were studying a blue-green azo dye which is formed by the combination of diazotated diethyl-saffranin and dimethylanilin, and which therefore is expressed by the formula



It was found that this substance has the power, somewhat like methylene blue, to stain the nerve endings of living (?) tissue organs,

whereas the corresponding dyes derived from saffranin, telosaffranin, and dimethyl-saffranin do not possess this property. Some time after this we received a second dyestuff, of unknown constitution, which possessed the same neurotropic properties, and we therefore at once assumed that this body also contained a diethylanilin radical. On inquiry of the manufacturer we found our conjecture verified. This staining experiment may perhaps afford valuable confirmation of the view expressed above concerning the function of the ethyl radical.

This synopsis will show that our actual knowledge concerning the relation between constitution and action is still in its very infancy. Hence the expectation to be able to construct new remedies of predetermined action on the basis of theoretical conceptions will probably have to be deferred for a long time. To the initiate the lack of sufficient positive knowledge is revealed by the inactivity which now characterizes a field once entered upon with so much promise. The innumerable remedies which have overwhelmed medicine in the past few years, of which only a few are of any value, and thus denote any real progress, have sufficed speedily to allay the original enthusiasm. A feeling of indifference has thus been engendered which is constantly being increased by the advertisements which are daily becoming more and more evident. Aside from these evils, however, this line of study is at present suffering especially from two other evils:

1. The habit, when a remedy has been partly accepted, of immediately following it with a dozen rivals of similar composition.
2. The exclusive preference given to remedies acting purely symptomatically, which are not true curative agents.

A change for the better will only then occur if pure biological points of view are adopted, i. e., if the initiative is transferred from the chemical to the biological laboratory. As physicians we must stop remaining content with the auxiliary rôle of counsel in these important questions. In this subject, our very own since time immemorial, we must insist on taking first place. Just now it is essential that we gain more general, biological conceptions, and it is therefore every one's duty to contribute his mite to the development of this therapy.

## II.

One of the main causes which has made an insight into the relation between constitution and action so difficult to obtain is to be found in the fact that these relations were considered to be much simpler than they really are, and in the further fact that purely chemical conceptions were applied arbitrarily to biological processes. In pure chemistry there is an abundance of material for observing the relations between physical properties and chemical constitution. In such a study it is first necessary to determine which properties, to follow Ostwald's terminology, are "additive" and which "constitutive" by nature.

The question arises what are the essential properties which are still found in the combinations. Evidently they are such as pertain to the *substance* of the elements and are independent of the *arrangement* of these. These properties accompany the elements in their combinations, assuming therein values which represent the sum of the values of the elements. In other words these are "additive" properties.

Real additive properties are not known apart from mass. The nearest approach to them are perhaps the specific heat of solid combinations, and in a less degree the refraction of organic substances and their property to occupy space. In these, however, another factor becomes evident, namely, the *arrangement* of the elements in their combinations. This factor is of paramount importance in determining such properties as color, boiling- and melting-point, form of crystals, etc. The properties which are under the mutual control of the nature of the elements and their arrangement are called "constitutive" properties. The extreme in this direction is made up of those properties which are no longer in any way dependent on the nature of the substances but only on their arrangement, these are called "colligative" properties.

To which group, then, do the properties of affinity, i.e., the power of elements to effect chemical reactions, belong? Evidently to the constitutive, for daily experience teaches us that the nature as well as the arrangement of the elements is a factor. Acetic acid, lactic acid, and glucose contain the same elements in the same proportions by weight, yet they manifest entirely different reacting capacities. Butyric acid and acetic ester are not only of the same con-

stitution but have the same molecular weight, yet their affinities are different.<sup>1</sup>

There is probably no doubt that those properties of organic substances which interest us as therapeutists are constitutive in nature.

R. Meyer has published a most interesting article on certain relations between fluorescence and chemical constitution. In this he calls attention to the fact that the relations between the color of chemical combinations and their constitution have not up to the present time been studied with the exactness with which characteristics less apparent have been examined, such as rotation and the refractive index. The reason for this is that the refractive index of a body is a definite number, the specific rotation an angle whose size can be exactly determined, whereas color is more qualitative in character, and, strictly speaking, is not a *physical* but a *physiological* characteristic. A body which possesses strong ultraviolet absorption bands is colorless to our eyes, yet it may appear colored to a visual organ differently constituted than ours. We see, therefore, that even in so conspicuous a property as color the physiological factor interferes with our gaining a clear insight into the relations existing between constitution and action. It will at once be conceded that this is true to a still greater degree in the complex processes which underlie pharmacological action.

But it is just because of this intermediate position that the chemistry of dyestuffs affords so good a point of vantage for our consideration. I may therefore perhaps be permitted to briefly outline what has thus far been learned concerning the relations between color and constitution, especially in view of the fact that I shall frequently have to touch on the biology of dyes in the succeeding chapters.

In 1868 C. Graebe and C. Liebermann demonstrated that color was in some way associated with a certain denser combination of the atoms. If this is overcome by the addition of hydrogen the color will disappear, the dye passing into the "leuco" combination (thus indigo into indigo white), out of which it can again be produced by oxidation.

A great advance was then made by O. N. Witt, who showed that the color properties of a dyestuff are due to the presence of a certain unsaturated group of atoms which he terms the color-producing or

<sup>1</sup> Ostwald, Grundriss der allgemeinen Chemie.

"chromophore" group. Concerning the details of the various types of chromophores I refer the reader to the admirable work of Nietzki. I may, however, say here that, as a rule, the action of the chromophore groups as such does not become manifest if the group is part of a molecule very poor in carbon atoms. Hence colored combinations are rare in the fatty series, they belong almost exclusively to the aromatic series (Nietzki). The presence of a chromophore group does not, however, by itself suffice to produce true dyes. Thus azobenzol, which possesses the chromophore azo group,  $N=N$ , is no dye, because it possesses no affinity for tissues. For this reason Nietzki terms azobenzol a "chromogen," i.e., a combination which becomes a true dye when suitable groups are introduced. Radicals which have the power to develop the nature of a dye are called "auxochrome" radicals (Witt). Thus far we know but two, namely, the OH group which produces dyes of an acid character, and the amido group which produces basic dyes. In contrast to this it is found that other salt-forming groups are not auxochrome. This holds true not only for acid complexes, such as the carboxyl group and the radical of sulpho acids, but also for certain basic radicals as  $NH_4$ ,  $CH_2NH_2$ ,  $CH_2N(CH_3)_2$  and  $OCH_2N(CH_3)_2$ .

From every chromogen, therefore, two series of dyes may be derived, acid and basic, each acid derivative having an analogous basic one. Thus

Acid	Basic
Oxyazobenzol .....	Amidoazobenzol
Dioxyazobenzol (resorcin yellow) .....	Diamidoazobenzol (chrysoidin)
Rosolic acid .....	Rosanilin
Thionol .....	Thionilin
Apocsafranin .....	Apocsafranin

If several similar auxochromes are introduced into a chromogen it will be found that up to a certain point the intensity of the shade and the affinity for the tissues increases with the number of groups introduced; thus, amidoazobenzol—yellow; diamidoazobenzol—orange; triamidoazobenzol—brown.

Witt's observations extended only to the question whether and under what conditions a body is colored. Nietzki went a step further and showed that the simplest azo bodies, as also all the most simply constituted dyes, possess a yellow color. He showed that the tint deepens not only with the increase in auxochrome groups just mentioned, but also with the accumulation of carbon atoms in

the molecule. In many cases the color thus passes through red into violet, in other cases it passes into brown. Besides this the chemistry of the rosanilin dyes furnishes many examples of change in tint through the introduction of substituting groups; thus, rosanilin: red, trimethylosanilin: red violet; hexamethylosanilin: blue violet, triphenylosanilin: blue.

I may add that in several cases these views have been applied also to bodies possessing physiological action. In cocaine, for example, the ester-like benzoyl radical,  $(CO \cdot C_6H_5)$ , undoubtedly represents the anesthesiophore group; the tertiary amin contained in the basic portion representing an analogue of the auxochrome group, and hence called auxotox. This is borne out by the fact, determined by me that cocaine loses its anesthetizing properties when through methylation the tertiary amin is converted into a quaternary ammonium base. Analogous to this is the fact that through complete methylation tertiary groups lose the property to act as auxochromes, for the ammonium radicals thus formed merely give rise to an increased solubility. Thus through the introduction of a methyl group, hexamethyl violet, which possesses three dimethylamido radicals, passes over into the soluble methyl green, which possesses two dimethylamido groups and one ammonium group. Hence methyl green is a triphenyl-methan dye which contains two dimethylamido groups as auxochromes. In this it is like malachite green, which it therefore matches entirely in tint.

The third portion of the cocaine molecule, the carboxymethyl group,  $COOCH_3$ , on the other hand, is probably of but little importance, as can be seen from the strong anesthetic action of benzoyl-pseudotropin, which does not possess this group.

### III.

Having thus briefly sketched some of the more important points concerning the relation between chemical constitution and action, I pass on the pharmacological side of the subject, in which, to be sure, the conditions are far more complex. It will be well to commence with a very simple example. We know a large number of poisons which through appropriate substitution are practically deprived of their deleterious action. As was shown by Aronson and myself, this is true, especially of the radicals of sulphuric and carbonic acids. Independently of us, Nencki came to the same conclusion.



Thus by allowing sulphuric acid to act on anilin, which, as is well known, is highly toxic, the toxicity is completely destroyed, for the resulting sulfanilic acid can be taken in large doses without injury. In like manner the amidobenzoic acids are non-toxic, so also the meta- and para-oxybenzoic acids derived from phenol, while the ortho isomer (salicylic acid) still exhibits the familiar toxic effects, although they are far less intense than those of phenol. These surprising results cannot be ascribed to purely chemical effects, as, for example, by assuming that the acid derivatives are more difficult to oxidize than the original substance and that they therefore do not abstract oxygen from the tissues. Certain observations, however, which I had made many years previously in connection with vital staining furnish a very simple explanation. I found that the power to stain gray nerve tissue is possessed by only a small number of dyes, and especially by certain basic dyes (chrysoidin, Bismarck brown, neutral red, phosphin, flavanilin, methylene blue), whereas of the acid dyes, in which OH constitutes the auxochrome group, only one, alizarin, possesses this property. All dyes which contained a sulphuric acid radical were absolutely negative, and I examined a very large number. What is especially significant is that even neurotropic stains lost this property entirely if sulfonic acids were introduced, a fact demonstrated in the flavanilin sulfonic acids, the alizarin sulfonic acids, and the sulfonic acids derived from methylene blue. From this it follows that the introduction of the above-mentioned acid group changes the distribution in the organism and causes especially a complete destruction of neurotropic properties. The central action of a poison is to be explained logically by an accumulation of the toxic substance in the central nervous system. Since, therefore, the central part of the toxic action has been completely destroyed by the introduction of a sulfonic acid radical we find that the reduction in toxicity is readily explained. It is obvious that under these conditions other toxic properties, which do not depend on the central nervous system may be preserved intact. Thus according to my observations the blood destructive properties of phenylhydrazin and benzidin are still present in their monosulfonic acids.<sup>1</sup>

<sup>1</sup> The action of these combinations is not as strong as the original substance, but this is probably due to the fact that the sulfonic acid radical (and even the neutral sulfonic radical) by itself reduces the toxic power of the amido group. This mitigating action explains why sulfanilic acid which is derived from anilin is no blood poison; this power of the sulfonic acid group, however,



From these considerations it is at once clear that there is a link between chemical constitution and pharmacodynamic action, namely, *the distribution in the organism*. In this we are dealing with a principle which has long been known, and which, I might say, is almost self-evident, but which nevertheless is clearly expounded in but few text-books on therapeutics (see Stodvis, de Buck, and especially H. Schulz).

Unfortunately we have been satisfied with a mere theoretical acknowledgment of this principle, and have practically made no efforts to gain a deeper insight into the laws governing this distribution. This is especially true of the new synthetic tendency, which labors exclusively for symptomatic effects and leaves questions concerning localization absolutely untouched. To my mind just this neglect is to blame for the insufficient progress thus far made, and I believe that new points of vantage can easily be gained if the *distributive* views are given greater prominence. In this connection I may call attention to the fact that through the application of the principle of localization, which I have attempted, new and promising paths have been opened up in the domain of bacteriology, although this subject was already beginning to become barren under the schematic application of the doctrines of immunity.

To be sure it must be admitted that there are enormous difficulties attending the determination of the distribution of chemical substances with the necessary degree of precision. We are here confronted with a problem whose solution is simple in only a few special cases. These we shall discuss in a moment. In the great majority of chemical compounds, however, only a combination of various methods gives us any definite knowledge.

Animal experiments, as such, do not give us complete information concerning the distribution in the organism, they only mark the regions most susceptible to the poison, and then usually only for those systems, such as the nervous or muscular system, in which disturbances of function are recognizable. The animal experiment, however, furnishes but little information concerning the processes in the vital parenchyma, for to these graphic or other ordinary physiological methods are inapplicable.

The assistance afforded by pure chemical analysis is very slight

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is insufficient to destroy the powerful  $\text{NH}\cdot\text{NH}_2$  group of phenylhydrazin, or the two amido groups of benardin.

It can be carried out exactly with only a very small number of readily determinable substances, hence primarily with inorganic combinations. Besides, the demonstration that a poison, for example arsenic, occurs in a certain organ, as the brain, is of little value, for this does not tell us what is really of the greatest importance, namely, the localization in the separate cell constituents of the various organs.

The pathological and histological findings are of far greater importance. To be sure, if one turns the pages of the text-books, one will not have very great hopes in this direction, for the same banal changes, fatty degeneration of the liver, nephritis, destruction of the blood, are always given. Nissl's investigations, however, demonstrated that exact histological studies on the central nervous system allow the points of attack to be recognized. He showed that certain poisonings always affected certain groups of ganglion cells. How fruitful these points of view may be was shown by the pretty investigations of Goldscheider, through which he showed that the motor ganglion cells had already suffered demonstrable lesions from tetanus poison at a time when even the slightest clinical symptoms were absent. In many other cases also, most valuable information may be furnished by minute histological investigations, in this connection I may mention that with cocaine I have found in mice an absolutely specific foam-like degeneration of the liver cells in a form which I have seen with no other substance. In general, I may add that the chronic poisonings extending over several days, and not the acute poisonings, are best suited for the demonstration of specific injuries to certain organs, a point which has already been emphasized by Nissl.

In my pharmacological investigations, which far antedate Nissl's publications, I have given this method special preference. I also described a method (*Deutsche med. Wochenschr.* 1890, No. 32) by which these otherwise laborious experiments can be carried out with ease. This method depends on feeding mice with biscuit which contains a certain amount of the substance in question. It is then very easy to find a dose which will kill the animals in the desired period of time.

Although the results of these anatomical-pathological investigations are most valuable, it cannot be gainsaid that through them one only discovers the injury to the most susceptible organs, but that the general distribution of a certain substance within the entire organism remains unknown.

In my opinion, however, this general distribution is a very important problem, for just these facts furnish the most valuable information concerning the chemical functions of the organs, and of the elements which compose them. At present this problem can only be solved by the employment of dyes whose distribution we can readily follow both macroscopically and microscopically. It is to be deplored that these investigations, which possess such a high didactic value should thus far have found so few adherents; they are only exceptionally studied and then for some particular purpose.

If rabbits are injected with dyes it will be found that even macroscopic study yields most interesting pictures. There are certain dyes, although not very common, which stain only a particular tissue, e.g. fat tissue; these are called "monotropic." Usually a dye possesses an affinity for a number of systems of organs, although frequently it then happens that one particular organ is stained in an especially conspicuous manner. Very often one finds that the maximum staining is in the kidney (especially in the cortex) and in the liver. Other dyes, such as acridinorange and dimethylamido-methylene blue, exhibit their stain particularly in the thyroid gland, still others, as dimethylphenylene green, stain especially the fat tissue, some, such as alizarin blue, the submaxillary gland, etc.

Alizarin blue, besides staining brain and kidneys, stains the submaxillary gland with especial intensity. As examples of polytropic stains we may mention neutral red and a basic dye, brilliant cresyl blue, for these stain the majority of body parenchyma intensely and apparently uniformly. It is particularly significant that the majority of basic dyes which stain the brain are also stored up by fat tissue. As we shall soon see neurotropism and lipotropism are related to one another.

The variation in the localization of dyes frequently corresponds to certain peculiarities in their excretion; the chief points of excretion are probably kidney cortex, liver, and intestine. In contrast to the great majority of dyes which, like methylene blue, fuchsin, alizarin, indigo carmine, and many others, gain access to the urinary secretions very easily, there are several which seem incapable of doing this and which therefore seem by preference to be excreted through the bile or through the intestinal pores. An example of this is benzopurpurin, a very large-moleculed cotton dye which is made from diazotated toluidin and naphylaminesulfonic acid<sup>1</sup>.

<sup>1</sup> It is possible that this phenomenon can be fully explained by this that we

Besides this, however, one could assume that analogous dyes also effect a loose combination with the blood albumin, which makes excretion through the kidney impossible. In that case the conditions would be analogous to those which we see with many metals, e.g. iron or lead, and to those which obtain in the excretion of a poisonous albuminous substance, renal, as they have been determined by investigations in the Pasteur Institute. None of the substances which occur in the circulation in the form of albumin combinations pass into the urine, since the albumin molecule is unable to pass through the intact kidney filter. In contrast to this, however, the intestinal glands or liver allow even these large-molecule substances to pass through.

The salivary glands do not play any important part in elimination, as is shown by the fact that with the majority of dyes the saliva is not at all colored, and with certain others, e.g. alizarin blue, is but slightly tinged. This is apparently because of the fact that the salivary glands are not well adapted to the secretion of substances with large molecular weights. In the excretion of substances of small molecular weights, however, they may play a prominent rôle, as can be seen from the behavior of various salts, e.g., potassium iodide, rodan combinations, and the salts of mercury. In the aromatic series it is particularly paraphenylenediamin, dimethylparaphenylenediamin, trihydroparaoxychinolin, and related substances, which are excreted through the submaxillary gland of rodents and there give rise to marked inflammatory changes (ordema, necrosis).

The least important rôle is that taken by the sweat glands. So far as I am aware the only dyes excreted on the body surface are those of the phosphin series, as is shown by Mannaberg's researches concerning the therapeutics of malarin.

Much greater significance, however, attaches to the possibility of exactly determining the distribution of the dyes by means of the microscope. I need only call to mind the vital staining of nerve endings by means of methylene blue, a procedure which has found

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are here dealing with large-molecule substances which are soluble with difficulty and which therefore must be regarded more like colloids. In contrast to methylene blue, methyl violet, and many other dyes, benzopurpurin is absolutely non-diffusible. According to the researches of Kraft (Bericht der deutsch. chem. Gesell. 1899) solutions of benzopurpurin (raising of the boiling-point) showed an apparent molecular weight of 3000 instead of 774 reckoned out from the formula.

extensive application in the histology of the nervous system. Then there are the wonderful vital stains which the majority of granules give with neutral red; and the beautiful stains of these same bodies which can be effected with brilliant cresyl blue (oxazin dye). I cannot here enter into still other interesting and important vital stains.

Besides this each stain possesses its own peculiar characteristics. Thus methylene blue, besides staining the nerve endings and a number of the most diverse granules, stains intensely the cell protoplasm of the islands of Langerhans of the pancreas, and, further, also muscle cells of a certain particular function, striped as well as smooth. I am practically convinced that in the vascular system certain muscle fibres which can be stained with methylene blue cause a marked narrowing and perhaps even a complete closure of the lumen after the manner of a ligature. These muscle fibres never form a continuous lining of the vessel wall but only occur singly and separated from one another by comparatively wide intervals. The uniform calibration of the tube would then fall to the lot of the evenly distributed muscle lining which takes no stain. We should thus have what is surely of great significance, namely, the fact that vessel calibration and vessel closure are two functions which are absolutely distinct anatomically and biologically. In a description so general in character as this one I cannot enter into still other interesting groups of dyes, e.g., those that stain nuclei vitally, etc.

Exactly the same differences which we have observed in the case of dyes manifest themselves if we introduce other kinds of substances into the body, it matters not whether they are well defined, organic or inorganic combinations, or whether they constitute chemically unknown and highly complex bacterial products. In general we shall probably have to assume that substances which are chemically well defined are to a great extent polytropic in character. In my studies with several substances readily demonstrable by means of color reactions and whose distribution can therefore readily be followed, I have convinced myself that the aromatic bases as a rule have an affinity for many different kinds of parenchyma. If in spite of this the clinical injury manifests itself in only one tissue, this in no way contradicts the polytropic character of these substances. It merely proves, what is really a matter of course, that among a number of tissues there are some that are particularly susceptible to an equal injury. To what extent other circumstances, such as saturation of

the tissues with oxygen, reaction of the tissues (metabolic in chromium poisoning), conditions of alkalinity, peculiarities of elimination, etc., affect the result in any given case cannot now be discussed. We find exactly the same conditions to hold with bacterial poisons. Tetanus poison, for example, as is shown by the experiments of Donitz, Roux, and others, is monotropic in highly susceptible animals, whereas in other animals, rabbits, pigeons, etc., the tetanus-binding groups are present not only in the brain but also in a number of other organs of less biological importance. This explains why, for instance, in guinea-pigs the lethal dose is the same whether the poison is injected subcutaneously or intracerebrally, whereas in the pigeon, and to a certain extent also in the rabbit, much larger doses are required for subcutaneous poisoning. Under these circumstances part of the poison is laid hold of by the body parenchyma and thus deflected from the endangered organs.

We may perhaps regard it as a matter of course, that these laws of mutual deflection play an important rôle in all polytropic substances, and that we shall gain a real insight into the action of drugs only if we regard this factor sufficiently. If, for instance, as is so often the case, a poison is both neurotropic and hypertropic, if the same amount of poison per kilo body weight is injected into a lean animal as into a very fat one, it is clear that the share of poison which falls upon the brain in the former case is much greater than in the latter.

#### IV

We now take up the question as to how this varied distribution occurs. As a rule the poisons reach the tissues through the circulation, and we shall therefore first study the influence of the vascular system on this distribution. A moment's consideration, however, shows that although the circulation may be the prerequisite, it can in no way be the cause of the varied distribution discussed above. According to the views held by the majority of investigators and also by me this localization in certain organs depends in every instance on causes within the tissues and not on the vascular distribution. For example, if in a case of jaundice we find that the brain shows not a trace of bilirubin coloration, while many other tissues, such as kidney, liver, etc., are saturated with bile pigment, this, in my opinion, is due to the chemistry of the brain substances. The brain lacks all such substances which attract bilirubin, that is to



say bilirubin is not neurotropic. In recent years a different view has been promulgated, especially by Biedl, who ascribes a decisive rôle in the distribution of poisons to the vessel wall. As a result of my own long experience with the greatest variety of substances I am unable to assume that the vascular endothelium as such exercises different functions in different organs, so that, for example, a liver capillary is permeable for certain substances which will not pass through other capillaries.<sup>1</sup>

On the other hand the vascular system plays a very important rôle in a different direction, as can be seen from the following striking example. Mice are fed according to my "lincit method" with derivatives of paraphenylenediamin (acetylparaphenylenediamin, thio-sulfonic acid and mercaptan of paraphenylenediamin). On autopsying the animals very peculiar changes are observed in the diaphragm. The parts surrounding the central tendon are stained intensely brown, while the peripheral portions are usually unstained. Frequently the margin of the stain is wavy and marked by a more intense coloration. At times I have observed similar changes in other muscular regions, namely, in those of the eye, larynx, and tongue. Macroscopical examination shows that this is not a case of infarct, but that there is apparently a uniform brown staining of the muscle areas in question. The cross striation is preserved intact, and a moderate degree of fatty degeneration is not infrequently observed. Usually also there is a certain amount of hyperæmia. We are not dealing with a derivative of hæmoglobin; on the contrary it is much more probable that we are dealing with a highly complex oxidation product of the paraphenylenediamin.<sup>2</sup>

The question which now arises is why, in this feeding, only part of the muscles, a very small part, show this vital staining.

It was soon seen that the groups of muscles affected were analogous in other respects. Thus with injections of methylene blue it

<sup>1</sup> It was especially gratifying to note that Bruno, as a result of the investigations which he made under the direction of H. Gutthieb, is also very skeptical regarding Biedl's views (*Deutsche med. Wochenschr.* 1899, No. 23).

<sup>2</sup> This assumption has subsequently been clearly confirmed by the work of Dr. Rehn (*Archiv. internat. de Pharmacodynamie*, Vol. VIII, p. 263). It was found in animals poisoned acutely with paraphenylenediamin that the muscles which were saturated with the poison assumed the typical brown color when brought in contact with air. I would also call attention to the fact that both paraphenylenediamin and paramidophenol are employed, by oxidation, for true brown and black dyes for hair and fur (Eosol dyes).

is just in these areas that the motor nerve endings take a more or less complete stain. In comparative pathology, now, we find this group in evidence for tritane axons by preference in diaphragm and the muscles of the eye and larynx.

These facts are very readily explained. In accordance with a principle discovered by Robert Mayer, the blood supply of the muscles is dependent on their biological importance. Muscles such as the diaphragm, which labor continuously and whose failure to act would constitute a marked disturbance of health are far better supplied with blood than others of less importance.

Naturally in this group of "most favored" muscles, corresponding to the greater supply of blood, there will also be a maximum supply of oxygen, foodstuffs and all other materials present in the circulation. Hence such a muscle cell will be more highly charged with oxygen and can therefore exert a more energetic oxidizing action, as is manifested in the brown staining with paraphenylenediamine. The staining of the muscle end plates is explained in exactly the same way, through the increased supply of methylene blue on the one hand, and the saturation with oxygen and the alkaline constitution of the nerve endings on the other.

An important principle governing the distribution of substances in the organism can be deduced for these experiments, namely, that myotropic and neurotropic substances can produce associated injury to certain systems solely through the character of the blood supply. It would, however, be wrong to assume that all muscle and nerve poisons must always injure only the most favored systems of muscles as described above. That would be disregarding the fact that the poisonous action is dependent not only on the supply of poisons but also on the capacity of the tissues to take up the poisons. A nerve ending of neutral or acid reaction will take up other substances (e.g. alizarin) than one of alkaline reaction (methylene blue). A muscle loaded with oxygen will combine certain substances and overcome their poisonous action, whereas that same poison will remain intact in muscle tissue deficient in oxygen.

I believe that the various nerve endings (motor, sensory, and secretory) are made up of the same chemical material. If, however, we consider the manifold and specialized actions of the alkaloids, for example, the very different actions of digitalis, curare, pilocarpin, and atropin, and if we ascribe the toxic action to an accumulation, we shall be forced to conclude that the nerve endings, though com-



posed of the same chemical substances, are subjected to different conditions in the various tissues, conditions which may possess a decisive influence. Foremost among these I regard variations in the reaction and in the degree of oxygen saturation to which I have already referred. As a result of my experiments in biological staining I assume that certain nerve endings, central and peripheral, are characterized by a particular complex of such determining factors, and that this "chemical milieu" represents the resultant of the normal physiological functions. Whether these views possess any heuristic value for the further development of the science, I do not know. For the present I shall content myself by remarking that the isolated disease of nerve or muscle apparatus, so far as it affects certain particular groups (lead paralysis, arsenic paralysis), is readily explained from this point of view. We shall have to assume the existence of just as many different types of nutrition as we can demonstrate different types of disease.

This brings me to a further question which concerns this distributive therapy, and that is whether it is possible simply by chemical means to change the type of distribution of a given substance. This question can readily be answered in the affirmative. If, for example, a frog is injected with methylene blue, the nerve endings, as is well known, will be stained in the living state. However, if an easily soluble acid dyestuff, e.g. orange-green, is added to the methylene blue solution so that a clear green solution results, it will be found that the injection of such a mixture no longer produces staining of the nerve endings. Hence we see that the conditions are entirely analogous to those which we find in the staining of dry preparations. The basic dyes by themselves stain nuclei, whereas the combination of basic dyes with acid dyes, which I introduced into histological technique under the name of "triacid dyes," lack this property to a greater or less degree. In both cases we are dealing with a distribution of the methylene blue between the acid dye and the tissue constituents. The tissues as well as the acid dyestuff have an affinity for the methylene blue. If the affinity of the tissues is greater, they will be stained blue; if that of the acid dye is the greater, the staining will not occur.<sup>1</sup>

<sup>1</sup> Naturally this phenomenon will occur consequently only in those cases in which the tissue substances possess an affinity for the base only and not for the acid dye. If the latter condition obtains the mixture of both components

In the deflection of methylene blue by means of orange we thus have presented a phenomenon which in its essential features reminds us of the mode of action of the antitoxins.

The opposite behavior, however, also occurs, namely, that the localization of a certain substance in a particular tissue becomes possible only through the simultaneous introduction of a second combination, even though the latter effects no union whatever with the first combination. Naturally these complicated phenomena can be demonstrated with certainty only by the aid of vital stainings, for in these can the microscopical distribution be positively determined. The following examples are the result of this method of investigation:

Bismark brown, the well known basic azo dye, exhibits a certain amount of neurotropy manifested especially in the staining of the gray matter of the brain. This affinity, however, is insufficient to give rise to a staining of the peripheral nerve endings in a frog, particularly a staining of the taste buds. If, however, a frog is injected with a mixture of methylene blue and Bismark brown it will be found that the terminal apparatus is stained a mixed shade. The blue very readily loses its color through reduction, and in a preparation mounted on a slide and sealed with a cover-glass the blue color can be seen to disappear rapidly, leaving only a pure brown stain.

The other example is still more striking. If a rabbit is infused with a solution of methylene blue, one always finds well-marked staining of the pancreas, due especially to a staining of the granules and protoplasm of the islands of Langerhans. In no case have I observed a staining of the nerve endings under these conditions. If, however, one adds certain dyestuffs of the triphenylmethane series to the fluid infused, dyes which in themselves do not stain the nerve endings, a truly beautiful staining of the nerve apparatus frequently occurs. In these and other similar cases I believe that we can only assume that the favoring substances cause a modification of the function of the apparatus in question, and that this carries with it a change in the "chemical milieu" defined above, and so in the absorbing power. It is possible that similar factors also play a certain rôle in many abnormal actions of drugs, especially in inherited or acquired hypersensitiveness.

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(i.e. the neutral stain) will come into play, a fact which is so well observed in the staining of the neutrophilic granules

## V.

The question now arises as to how we conceive this selection of the tissues to occur. It is very probable *a priori* that we are dealing with chemical affinities in the widest meaning of the term. We must, however, discuss in detail the nature of these affinities. In this, I must emphasize, we are dealing primarily with substances which, like the various natural and artificial drugs, are foreign to the body, not with foodstuffs capable of assimilation. The latter will be treated by themselves subsequently.

The simplest case is that in which the organism is injected with indifferent substances, neither acid nor basic in character, to which, corresponding to their constitution, we can ascribe no great chemical affinities, but which nevertheless exert marked and often highly toxic effects. In this category belong especially the various hydrocarbons, e.g. toluol, benzol; a number of ketones, such as acetophenon, many sulfones, which are characterized by their chemical indifference, also various kinds of ethers, alcohols, and a large number of other narcotics. The best opinion seems to be that in these cases no direct chemical affinities come into play on the part of the organism, and that the molecule is always present in the tissue constituents unchanged and chemically uncombined. That is to say, the phenomenon is one of contact action. In spite of this it can readily be shown that all these compounds possess a typical localization in the tissues, the cause of which we shall soon discuss.

First, however, I should like to say a few words concerning the historical side of this question. The idea that chemical substances can act solely through contact was first affirmed many years ago, thus by Buchheim in 1859, Schmiedeberg in 1883, Harnack in 1883, and by Geppert. The latter's investigations may be found in the *Zeitschrift für klin. Medizin*, Vol. XV, and deal with the nature of prussic-acid poisoning. He showed that in this highly interesting case the hydrocyanic acid acts as such. He explained the result of the toxic action in the following manner:

"We know that chemical processes are retarded simply through the presence of minimal amounts of prussic acid. Thus picric acid does not yield up its oxygen to formic acid under conditions otherwise favorable if even a minimal amount of prussic acid is present. It is quite natural, I suppose, that in the poisoned organism, highly

oxidized substances (the analogues of iodic acid) are no longer able to yield up their oxygen to oxidizable combinations when prussic acid is present. (One must think of these highly oxidized substances as transmitters or carriers of oxygen.) Prussic acid poisoning is therefore an internal suffocation of the organs."

This discovery of contact action constituted the first step toward penetrating the mystery of the action of drugs. This, however, afforded no explanation as to why the substances mentioned exhibited an elective action. That was because the link was missing which, according to modern views, is absolutely indispensable, namely the connection between action and distribution in the tissues. I think I am justified in claiming to be the first to recognize the right path, for in 1887, in my article on "The Therapeutic Significance of the Substituting Sulphuric Acid Group" (*Therap. Monatshefte*, March, 1887), I demonstrated that neurotropic stains are deprived of this property on the addition of the sulfonic acid group. Even at that time I compared the localization of the dyes and of the alkaloids in the brain with the principle of the shaking-out procedure devised by Stas-Otto, expressing myself as follows:

"The principle of 'shaking-out' persons devised by Stas-Otto depends on the fact that basic substances, e.g. alkaloids, etc., are generally firmly combined in acid solutions, and hence extracted with difficulty, whereas the same substances can readily be shaken out of alkaline solutions. Acid substances, of course, exhibit exactly the opposite behavior; they are held back by alkaline media, but readily given up by acid media. If we apply these experiences to the question under discussion we can readily understand why basic dyes (which are not held back by the blood through any chemical affinities) are especially laid hold of by the brain, whereas the acid dyes and the sulfonic acids (which are bound by alkalies of the blood to form salts, and are thus anchored, as it were) show exactly the opposite behavior."

Besides this I showed that fat tissue behaves like the brain, for a large part of the substances taken up by the brain are taken up also by the fat tissue. In 1891 this question received a fresh impetus, for Hofmeister, Pohl, and also Spuro, called attention to the significance of loose combinations which could readily be dissociated. Thus in 1891 Pohl showed that the ability of the red blood-cells to take up chloroform, a fact which Schmiedeberg had demonstrated in 1867, was due to the cholesterol and lecithin which

the cells contain. Both substances can be shaken out with chloroform. He also referred the union of chloroform in the brain to similar fat-like bodies in that organ, as I have done for the coloring matter of the alkaloids. A basis was thus secured from which to study the action of the above-mentioned substances in the brain. These substances, it will be seen, are most all readily soluble in fats and fat-like bodies, corresponding to their physico-chemical nature.<sup>1</sup>

The conditions, however, were far more complex in the large number of bodies which, like many medicinal substances (e.g., the antipyretics), and the most varied basic substances (among these the alkaloids), phenols, aldehydes, and many others in contrast to the indifferent bodies, do not seem incapable of combining synthetically with the tissues. In numerous articles Low assumes that most of the bodies in question are able to unite synthetically with constituents of the cell or with the living protoplasm. It is obvious that we must assume the protoplasm to contain many different kinds of atomic groups possessing very strong affinities, and it was certainly very plausible when Low ascribed a leading rôle in the phenomena of poisoning, to groups so well able to act. His experiments and researches lead him to conclude that in the cell it is particularly aldehyde groups or labile amino groups which play this anchoring or grasping rôle. According to Low all substances which can combine with these two radicals are poisons for the protoplasm, the greater the affinity the stronger the poisonous action.

Against this view of a substituting action of the poisons a large number of easily verified facts can be brought forward. If benzaldehyde and aniline (or phenylhydrazin, etc.) are mixed, the two substances will condense to form a new substance, benzylidenaniline, water separating at the same time. This benzylidenaniline is a single

<sup>1</sup> It is impossible to do more than refer to the great advances made since my address, especially through the labors of Hans Meyer and Overton. In three studies on the theory of alcohol narcotics (*Archiv f. experim. Pathologie* 1899-1901), Meyer has shown in the most exact manner for a large number of chemical substances that the mode of action of the indifferent narcotics is not dependent on their other chemical properties but is governed exclusively by the partition coefficient which determines their distribution among water and certain fat-like substances (brain and nerve fat). If Overton came to the same conclusion regarding the causal relation between solubility in fat and narcotic action. His investigations, which have been gathered together in a work entitled "*Studien über die Narkose*," Jena, 1901, dealt especially with vegetable cells and small animals present in the fluid.

body which does not give up either anilin or benzaldehyde to indifferent solvents. It requires chemical splitting in order to form the two original substances.

In this way the question can very readily be decided whether or not a certain substance is anchored to a cell synthetically, for the material in question need simply be treated with indifferent solvents possessing strong extractive properties (alcohol, ether, etc.). If animals are injected with the most varied poisons, alkaloids, phenols, anilin, dimethylparaphenyldiamin, antipyrin, thallin, etc., and if one waits until the distribution is completed (which usually occurs in a moment), it is easy to extract the unchanged poison by means of suitable methods of extraction, and, provided the substance is easily detected, like thallin or dimethylparaphenyldiamin, to discover it in the tissues by means of staining reactions. Naturally these experiments are carried out most strikingly with dyestuffs, for in these the extractive decolorization of the methylene-blue brain cortex or of the fuchsin kidney can very easily be followed.

The experiments with dyestuffs furnish still another argument against a process of substitution. In the basic dyes when one or several amido groups are replaced by aldehyde radicals a change in color often takes place. Thus by means of aldehyde, fuchsin red is made to yield violet dyes. In accordance with Löw's theory one would have been led to suppose that when suitable dyestuffs were employed a change of color due to substitution should occur in some case or other and in some organ or other. In spite of experiments specially devised for the purpose I have never observed this to occur, either with dyestuffs which, like those mentioned above, unite with aldehyde, or with certain basic dyes (e.g., the azonium base which Kehrman produces from safranin) which take up amido radicals of the most varied kinds and cause an intensification and change of the color characteristics.

Many other reasons can be adduced which speak against the correctness of Löw's theory. I may merely mention the transitory character of the action, a point which is so often noted, especially in the alkaloids; furthermore, in the case of many drugs, the rapid elimination, which argues against a firm synthetic combination; another fact, one which may perhaps be of practical importance, is this: that in the construction of new therapeutic substances efforts were directed particularly to the elimination (by appropriate substitution) of groups which could effect syntheses. This is the case,

for example, with phenacetin, in which by the introduction of the methyl radical and of the acetyl group the powerful OH and  $\text{NH}_2$  groups of paraamidophenol are occupied.

All this has led me to conclude positively that Low's theory of the substituting action of therapeutic substances is untenable.

By this I do not in the least wish to say that groups capable of reacting, such as Low presupposes to exist in the living protoplasm, cannot occur there. It must be borne in mind, however, that condensation phenomena are not produced merely by the presence of two substances capable of condensing, but that the condensing affinity must usually first be increased through appropriate means, such as increase of temperature, the addition of substances abstracting water, etc. Even in the practice of the synthetic chemist, who allows the substances to act on one another either directly or in concentrated solutions, such direct condensations are not especially frequent. The number of these, however, is still more limited if the synthesis is to occur under conditions corresponding to those in the living organism, i.e. in dilute solutions, at low temperature and in the absence of suitable auxiliary substances. Dimethylamidoaldehyde unites with indol, for example, even in dilute solutions, at room temperature, forming a red dye, but only when the solution contains small amounts of free mineral acid. If this is absent, or if the solution is even faintly alkaline, no combination of any kind occurs.

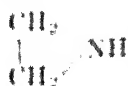
## VI.

These considerations lead at once to the view that in certain cases apparently it still is possible to effect a substitution within the organism by the introduction of chemical substances. In order to accomplish such a synthesis the selection of suitable substances will be prerequisite, and these substances must be of such a chemical constitution that they can exert chemical influences of the most powerful kind. I have made extensive experiments with many hundreds of different combinations, and in all of these I have only discovered one substance to which I am inclined to ascribe such a substituting action on protoplasm. This substance, emylamin, discovered by Gabriel and described by him in a masterly manner, is formed by abstracting bromine from bromethylamine by means of potassium





Since then, however, Marekwald has positively shown (1900-1901) that this substance cannot, as was at first supposed, contain a double bond (ethylene combination), for it does not reduce permanganate at ordinary temperature nor take up bromine. It can therefore only possess the constitution of a dimethylenimin:



In view of this a complete analogy exists between the ethylenimin and the ethylenoxid:



In conformity with Bayer's tension theory we must ascribe an extraordinary tension to the three-sided ring contained in the dimethylenimin. This manifests itself also in the fact that this substance shows a marked tendency, through the addition of acid radicals and the breaking of the ring, to pass over into a substituted ethylamin of the chain series. Thus, as Gabriel showed, HCl is added with the formation of chloroethylamin, and sulphurous acid with the formation of taurin. These reactions proceed with great energy, as is shown by the fact that even in dilute watery solutions of the freshly prepared hydrochloride an alkaline reaction develops within a few minutes, due to the formation of free chloroethylamin which reacts alkaline.

Ethylenoxid behaves in an analogous manner. This is shown in surprising fashion by the fact that this neutral body precipitates magnesia out of chloromagnesium, iron oxide out of iron chloride, entirely after the manner of free alkalis. In doing so it adds the acid radical and becomes transformed into chloroethylalcohol.

These two substances, ethylenimin and ethylenoxid, are highly toxic combinations as has been shown by the researches of Levaditi and myself. The pathological changes excited by dimethylenimin

<sup>1</sup> I have taken the liberty of somewhat modifying the text of this chapter in accordance with the positive advance of our knowledge, which we owe to the labors of Marekwald.



are especially interesting. Administered to a great variety of animals (mouse, rabbit, dog, goat, guinea pig, rat) in doses which cause death after 1½ to 2 days or more, this substance causes total necrosis of the kidney papilla. In the rabbit Levaditi found, besides this, marked changes extending from the pelvis of the ureter to the urethra, and consisting of necrosis of the lining epithelium, hemorrhages, and oedema. (*Archives internat. de pharmacodynamie*, Vol. VIII, 1901.)

Every one who has learned to know these changes (changes absolutely unique in pathology) will be forced to the assumption that this localization is dependent on a direct attack of the vinylum on the affected epithelia, an ethyl amide group entering the protoplasmic molecule. This assumption is supported by the fact, that only the active three-sided ring is able to produce this phenomenon, not the ethylene combination ( $\text{CH}_2 = \text{CH}_2$ ), furthermore, the fact that neurin (triethylvinylammonium hydroxid) which can be obtained by an exhaustive methylation of the dimethylenum, acts in an entirely different manner. That we are dealing with a typical ethylene combination is shown by the behavior toward bromine and permanganate of potash.

It has, of course, long been known that neurin is a highly toxic substance. Aside from its clinical toxicological mode of action it is characterized by an exceedingly evanescent action in contrast to dimethylenum. The toxic phenomena develop rapidly and disappear equally so without leaving behind any permanent injuries, especially destruction of the papilla. In contrast to this, vinylum is characterized by a slowly developing action, which in small doses may show several hours' incubation period and leaves the organism permanently damaged. I have compared this action with that of several other compounds which I have studied; thus camphylum, which according to Duden has the composition



allylamin with a double bond (ethylene radical):



and propargylamin, which contains the acetylen group,



All of these substances were found to possess the evanescent general symptoms together with an absence of permanent organic injuries. Hence I believe that the chemical avidity of the double and triple combinations is insufficient to effect substitutive reactions with the protoplasm. I am strengthened in this view by the

fact that prussic acid, which owing to its threefold combination  $\begin{array}{c} \text{CH} \\ ||| \\ \text{N} \end{array}$  can be classed with the most active substances known to chemistry, is nevertheless not anchored in the animal body as can be seen from Geppert's findings already referred to.

If we consider that substances which possess double or triple bonds are usually much more poisonous than the corresponding saturated combinations,<sup>1</sup> and if we bear the above considerations in mind, we shall ascribe this increased toxicity not to a combining capacity but to the fact that the unsaturated groups possess *auxiliary* properties, i.e., that they are able to increase the toxicity when they enter into complexes which in themselves already possess certain toxic properties.

I must emphasize the fact that all observations thus far made are only to be applied to organic substances foreign to the body. We must, however, assume that all substances which enter into the construction of the protoplasm are chemically fixed by the protoplasm. A distinction has always been made between substances capable of assimilation, which serve the nutrition and enter into a permanent combination with the protoplasm, and substances foreign to the body. No one believes that quinine and similar substances are assimilated, i.e., enter into the composition of the protoplasm. The foodstuffs, however, are bound in the cell, and this union must be regarded as a chemical one. The sugar molecule cannot be ab-

<sup>1</sup> Neurin is twenty times as toxic as cholin (trimethylethylammonium hydroxide); allyl alcohol fifty times more toxic than propyl alcohol, cf. also Low, *Naturliches System der Giltwirkungen* 1893, page 95.

structed from the cells with water, it must first be split off by means of acids in order to set it free. Such a chemical union, however, just as every synthesis, presupposes the presence of two combining groups of maximal chemical affinity which are fitted to one another. These groups in the cell which anchor foodstuffs I term "side chains" or "receptors," the combining group of the food molecule the "haptophore group." Hence I assume that the living protoplasm possesses a large number of such "side chains" and that these in virtue of their chemical constitution are able to anchor the greatest variety of foodstuffs. In this way the cell's metabolism is made possible.

This view of the constitution of the protoplasmic molecule has made it possible to get a much clearer insight into the action of the toxins and into the hitherto mysterious phenomenon, the formation of antibodies. I assume that the toxins, just like the food molecules, possess a particular haptophore group, which, by fitting into the receptor of the cell, gives rise to the poisonous action. Tying this receptor out of action causes a formation of new receptors to replace it, and these are finally thrust off into the blood. The receptors thus present in the blood constitute the antibodies. This theory, known as the "side-chain theory," has proved its worth in the hands of numerous investigators, for by its means the manifold reactions of immunity are all led back to the simplest processes of cellular life.<sup>1</sup>

Hence I assume the presence of a haptophore group only in such combinations which, like the foodstuffs, enter into the substance of the protoplasm, or which, like the large number of poisonous and non-poisonous metabolic products of living cells, effect a union similar to that of the foodstuffs.

The marked difference between the two classes of substances becomes plainly evident by the fact that only those substances possessing haptophore groups are able to excite the production of antibodies through immunization. And despite the most painstaking efforts neither other investigators nor I have ever succeeded in producing any appreciable production of antibody with alkalis, phosphates, or drugs of well-known chemical constitution.

<sup>1</sup> I content myself here with these brief remarks and refer the reader to my more recent detailed articles: 1. On Immunity, etc., *Comptes Rendus Acad. Sci. Proceedings of the Royal Soc.*, Vol. 66, 1900. 2. *Neuroendocrinologie und Anämie*, in Nothnagel's *Handbuch*, Vol. VIII, 1901, pages 525 et seq. 3. *Die Schutzstoffe des Blutes*, page 304 of this volume.

## VII.

In the case of the chemically defined poisons, drugs, and dyes discussed above, incorporation into the protoplasmic molecule does not, barring a few exceptions, take place by means of synthesis. Since, however, almost the greater part of all substances foreign to the body exhibit a typical selective action in the tissues, it becomes necessary to study the reasons for this action. Here again we shall do best to begin with a consideration of the phenomena which takes place in staining reactions. A cotton fibre placed in a dilution of picric acid of one to a million takes up the dye, becoming intensely stained. Methylene blue introduced *intra vitam* into the organism is taken up by the nerve endings. In poisoning by alkalooids certain nerve centres may react specifically and alone. All of these phenomena are obviously analogous in their nature. It seems necessary, therefore, to discuss briefly the views held concerning the nature of the staining process. The purely mechanical conception which refers it all to physical processes, such as surface attraction and absorption, can probably be discarded for the staining of substances in general. This leaves only two other explanations, either of which may be the correct one for certain cases.

The first of these, maintained particularly by Kuercht, proceeds from the assumption that certain constituents of the fibre substance form with the dye insoluble salt-like combinations usually termed laky combinations. This conception is supported by the fact that by treatment with alkalis an acid can be obtained—lanugine acid derived from wool, and nucleic acid from nuclear substances—which possesses the property of precipitating the salts of basic dyestuffs even out of very dilute solutions. Analogous conditions are found to a great extent in vital stainings. I need only remind the reader of the investigations of Pfeiffer. These show that in the vital staining of plant-cells one can frequently observe that the staining is due to conspicuous granules of the almost insoluble tannate of methylene blue. Naturally in the higher animals secretion substances present in the cells and constituting precipitants which form laky combinations can play a part in localization.

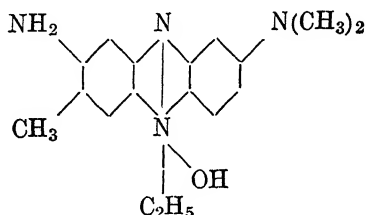
The second theory, one which associates the staining process with the phenomenon of solid solutions, we owe to the researches of O. N. Witt. This investigator starts with the fact that silk dyed

with rhodamin exhibits a beautiful fluorescence. Rhodamin itself, however, shows fluorescence only when in solution; when in the dry state, even in the finest possible form it merely shows a pure red color. Because of this fluorescence Watt assumes that the dye forms a homogeneous mixture with the fibres of the silk, i.e., that it is in the form of a solution. Since the fibre, however, is a solid substance this solution must be what Van't Hoff terms a "solid solution." We know that the same dye often produces different tints in cases of kinds of fibres. This is analogous to the fact that the same substance often dissolves in different solvents in entirely different tints, as in the case, for example, with resine. Watt therefore believes that the process of staining proceeds exactly the same as the distribution of a substance in two different solvents. Thus, if we spread cambric in water, we find that we can shake all the aniline over with ether, because the solvent power of the ether is greater than that of water. In the staining process such a vast difference in solvent power shows itself by the fact that the material introduced contains a large part of the staining bath. If, however, the difference in solvent power is less than this, e.g., in the combination water-ether and resins, we should find that the resorcin is distributed between both fluids in accordance with a law of distribution which can be figured out mathematically for every case. In dyeing this type corresponds to the dyes which are said to "take" poorly. In these the staining bath does not become exhausted under ordinary conditions. Exhaustion can be effected only through the addition of certain substances which limit solution (salt dyes, etc.).

In the introductory chapter I have already mentioned that all neurotropic and lipotropic substances lose the property to stain brain substance and fat by the introduction of the sulfonic acid radical. If these substances are examined in a test tube it is found that this substitution has caused them to lose also the solubility in ether or in fats. Thus, although flavanilin is easily taken up by ether from an alkaline solution, not a trace of flavanilinsulfonic acid is taken up. Another interesting case may be mentioned, one which concerns staining with neutral red. This has the following formula:



This substance has the property of staining the granules of cells most intensely, and the same holds true of a number of derivatives, e.g. violet dimethyl neutral red, in which the two hydrogens of the second amido group are replaced by two methyl groups; further, also, the golden-red diamidophenazin:



In contrast to this, however, the combination in which one of the central amin radicals contains an ethyl group which gives to the group the character of an ammonium base, is absolutely unable to effect the staining. All phenazin derivatives which stain granules can be completely shaken out of weak alkaline solutions by means of ether, whereas not even a trace of the ammonium base belonging to the safranin series is thus taken up by the ether.

A very intimate connection, however, exists between solubility in the test-tube and ability to be absorbed in the organism, a connection which I observed as long as fifteen years ago. Hence we must assume that certain fat-like substances of the nervous system as well as the fat of fat cells possess a high solvent power by means of which these substances are anchored or stored up in the tissue in question, just as the alkaloids are taken up by the ether in the Stas-Otto procedure.<sup>1</sup>

If we bear in mind not only the extraordinary multiplicity of substances foreign to the body, but also the varying chemistry of the tissues which make up the organism, we shall not expect that a single principle can be rigidly applied to the phenomenon of

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<sup>1</sup>This behavior has been studied especially by Overton. He terms the substances of the brain which serve as extracting agents "lipoids." Chief among these are cholesterin and lecithin. Among the alkaloids Overton distinguishes feebly basic and more strongly basic substances. The former can be shaken out—for example, the indifferent narcotics; whereas the more strongly basic unite with constituents of the cell to form salt-like combinations which are very easily dissociated. According to Overton's conception, therefore Knecht's explanation would apply at one time and Witt's at another.

selective action. For a large number of substances which localize in fat or fat-like bodies during life, it will probably be difficult to prove whether a pure shaking-out process occurs or a formation of but slightly soluble salts.

Furthermore, both processes may occur together, as Knecht assumes in dyeing, the lake-forming components being contained in the tissues in the intimate molecular mixture characteristic of solid solutions. In that case the resulting selective action will be due to a combination of salt formation and solid solution. In many instances, however, it will be extremely difficult to decide whether one is dealing with solid solution or salt or double-salt formation, especially since chemistry often finds it impossible to decide this question in the case of pure bodies. This is seen, for example, in the study of mixed crystals which are looked upon mostly as crystalline solutions.<sup>1</sup>

In any case we see that even without the intervention of a chemico-synthetic union the conditions necessary for a selective storage of a substance in the organism are present and are sufficient both in extent and in variety.<sup>2</sup> That these conditions in the case of the salt-like combinations are essentially chemical in nature is self-evident; in the case of the solid solution the enormous mass of evidence which I have merely touched makes this extremely probable. If we regard the principles governing distribution in the organism from these standpoints we shall no longer be surprised that in the localization

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<sup>1</sup> If two combinations of somewhat similar chemical constitution (for example, benzole and pyridin; stilben, benzylidenanilin, and azobenzole; fluoren and diphenylenoxid) form mixed crystals with each other, one can readily comprehend this in view of their close chemical relationship, and can ascribe it to "isomorphogenous" groups. Frequently, however, substances crystallize together which exhibit the greatest divergence in the configuration of their molecules, as, for example, phenol and urea, chloroform and salicylid, triphenylmethan and benzol. The crystalline fiery-colored combinations which pteric acid is able to effect with a large number of hydrocarbons are especially important. Certain investigations concerning the basic properties of oxygen (Baeyer) and of carbon (Kehrman and Baeyer) seem to show that such crystallizations, as, for instance, of ferrohydrocyanic acid with ether, etc., are analogous of salt formation.

<sup>2</sup> I must here refer the reader to the extremely interesting investigations of Spiro (*Über physikalische und physiologische Selection, Habilitationsschrift, Strassburg 1897*). In these, although starting from entirely different standpoints, the author reaches many of the views held by me. At the time of my address I was unaware of this study, as it is not to be had in the bookshops.

of substances foreign to the body synthetic processes play practically no rôle whatever. If we take methylene blue as an example, we see at once that we can easily find a large number of different fluids which are able to shake it out. On the other hand, we know of a large number of acids, like picric acid, phosphomolybdic acid, hyper-sulphuric acid, which are able to precipitate the methylene blue in insoluble form even out of very dilute solutions. This dyestuff, however, is practically useless for synthetic processes; all the efforts of the chemists to introduce other groups into the completed molecules (with one exception, nitro-methylene blue) have absolutely failed. When we stop to consider that in such chemical procedures the strongest possible agents can be used, sulphuric acid, high temperatures, etc., we shall at once see that methylene blue cannot at all be synthetically bound in the organism. The extensive distribution of methylene blue, however, is very easily explained by the plentiful opportunities offered for localization.

Synthetic processes, such as occur in the absorption of foodstuffs, in assimilation, and in the growth of living matter, are connected with the existence of certain chemical groups, the "receptors." These receptors are able to synthesize with fitting haptophore groups of the foodstuffs or of the toxins, the two groups fitting specifically to each other (like lock and key: E. Fischer). The eagerness with which the living protoplasm lays hold of the foodstuff which it requires is in marked contrast to the manner in which it resists taking up substances foreign to itself. This was observed even in the beginning of histology, for at that time it was regarded as an axiom that living cells could not possibly be stained. Gerlach, for example, had shown that an amoeba does not take up any coloring matter from a solution of carmine, whereas it stains immediately when it is dead. Since then, to be sure, largely through my efforts, we have come to know a number of important vital stains (neutral red, methylene blue, brilliant cresyl blue), but closer analysis of these phenomena have shown that that which can be demonstrated in the living cell by the various dyes is not the functioning protoplasm but its lifeless (paraplastic) surrounding medium and the granules, etc., present therein. In this point I agree entirely with Galeotti.



## VIII.

What practical conclusions can be drawn from these considerations? We see that drugs, such as the majority of narcotics—in fact the large number of neurotropic and lipotropic substances—become localized through a shaking-out process. It follows from what has already been said that only such substances can be anchored at any particular part of the organism which fit into the molecule of the recipient combination as a piece of mosaic fits into a certain pattern. Such configurations, however, are not confined to a single substance, but usually include a large group of related substances. In this connection the investigations which Einhorn<sup>1</sup> and I made concerning the action of cocaine are most important.

Cocaine is a derivative of ecgonin, whose molecule contains two groups differing in function: a hydroxyl group, which combines with acid radicals, and a carboxyl group, which forms esters with alcohol radicals. All derivatives of ecgonin in which both groups are thus occupied represent bodies of the cocaine series. Thus in the cocaine ordinarily used in medicine the acid radical is that of benzoic acid, the ester former is a methyl group. By means of the methods of modern chemistry it has been possible to introduce the greatest variety of radicals into ecgonin, leading to the formation of a large number of homologous substances. It was soon found that the substitution of other alcohol radicals, such as ethyl, propyl, etc., for the methyl radical did not cause the least change in the physiological effects of the cocaine, as Falk proved. On the other hand, the acid radical is of prime importance for the anæsthetic action of the cocaine. Poulsen, Liebreich, and myself studied the various cocaines with other acid radicals (cinnamyl cocaine, phenacetyl cocaine, valeryl cocaine, phthalyl cocaine) and found only one, the phenylacetic acid derivative, which possessed even feeble anæsthetic properties. As a result of these toxicological experiences one could have assumed that this benzoyl cocaine was in every way unlike all other acid derivatives. But this is not the case, for I was able to show that so far as another toxic action is concerned all of the various cocaines show the same

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<sup>1</sup> Einhorn is one of the best authorities on alkaloids known to me. The studies referred to, appear in the *Deutsche med. Wochens.* 1890, No. 32, and in *Berichte der deutschen chem. Gesellschaft* 1894, Vol. 27, page 1870.

behavior, namely, in mice they all produce a peculiar foam-like degeneration of the liver-cells which I have observed only in substances belonging to this series. From this it follows that all bodies of the cocaine series are alike so far as the liver is concerned. Considering that the substances which precipitate and dissolve these bodies are the same and that the liver findings are identical, we may perhaps assume that all cocaines are taken up by the liver in the same way and therefore probably also by the other parenchyma. And since the benzoyl derivative is the only one which possesses anæsthetic action we shall have to assume that the rest of the molecule is only the carrier which brings the benzoic acid radical to the proper place. (The anæsthesiophore character of this group had already been made very probable by the earlier investigations of Filehne.) Let us go back to our illustration of the mosaic in order to get this idea clearly before us. In order for a piece to help complete a given figure it is first necessary that it possess a *particular form*, but in order that the pattern be really completed the piece must also possess certain *material properties*, such as hardness, color, lustre, etc. It will be one of the problems of the future to extend our knowledge concerning the active toxophore groups.

The first fundamental experiments in this direction were made by Ladenburg, who showed that the two substances obtained on splitting atropin, namely, tropin and tropic acid, could readily be recombined and the atropin molecule thus be reconstructed. As a result of this demonstration that atropin represents an acid ester of tropin it was possible to produce a number of homologous combinations, Ladenburg's "tropeins," e.g., benzyltropin, salicyltropin, phenylglycoltropin (homatropin). A comparative study of the these substances showed that for mydriatic purposes aromatic oxyacids were the most favorable—and especially those in which the hydroxyl is in aliphatic combination, as in tropic acid and phenylglycolic acid.

In cocaine, Einhorn and I attempted to determine the function of the benzoyl group by introducing various side-chains. It was found that the introduction of a nitro group in the meta position had a marked influence on the anæsthetizing property of cocaine without preventing the injurious action on parenchyma described above. The introduction of a hydroxyl group in the same place acted still more strongly in this direction, for the anæsthetizing property had disappeared, the toxic action on the liver decreased. Meta-amido cocaine was entirely inert.

What was extremely interesting was the fact that by the introduction of suitable radicals into this inert amido cocaine the alkaloidal action could be restored. Thus when acetyl and benzoyl groups are introduced into amido cocaine, cocaines are formed which, although they are not anæsthetic, again possess this property of acting on the liver. It is especially interesting, however, that the cocaine urethane obtained by the action of chlorcarbonic acid on amido cocaine again acts anæsthetically, in fact much more so than the original cocaine. That is to say, if we nitrify cocaine, reduce it to amido cocaine, and finally condense it to a urethane, we find that the anæsthesiophore group is first diminished in power, then its action is entirely lost, and finally heightened. We already know the function of the toxophore group in a number of alkaloids, in atropin for a single group, in strychnine for two. If only we had a deeper insight into this function we might hope by means of substitutive action on the toxophore groups (such as Einhorn and I have carried out on the benzoic acid radical of cocaine) to modify the action of the alkaloids to suit our purpose.

In the synthetic field of pharmacology, however, a knowledge of the groupings on which the selective distribution in the organs depends would appear to be far more important. In the case of foodstuffs and toxins I assume that the union is effected by a single definite group, the "haptophore" group. Substances foreign to the body, as already explained, lack such a single group and the laws of distribution in the organism are dependent on the combined action of the separate components. In their distribution, therefore, the *entire constitution* of the substance is the deciding factor. This we have seen to be true with substances belonging to one group. Within this group type, as we have described it in detail with the cocaine series, modifications of the separate components can then be made within wide limits. Starting from this point of view we obtain a new method of synthetic-chemical pharmacology. If one is desirous of studying organ therapy in this sense it will be necessary first to hunt up bodies which possess a particular affinity for a certain organ. Having found such bodies one can then use them, so to speak, as a carrier by which to bring therapeutically active groups to the organ in question. It is self-evident that in the selection of these groups one is bound by definite limits; so also is the fact that all substituting groups which themselves influence the distributive character (e.g. acid radicals) must be avoided. All these are problems which ex-

tend far beyond the powers of single individuals and make it desirable that chemists and pharmacologists work together in some definite plan. That is one reason why I have gone into such detail concerning my views on the connection between constitution, distribution in the organs, and pharmacological action. I shall indeed be happy if these views, the gradual development of ten years of study, will advance the study of pharmacology.

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TRANSLATOR'S NOTE.—See also the recently published study by Bechhold and Ehrlich on the relation of chemical constitution to disinfecting power. (*Zeitschrift für physiol. Chemie*, Vol. XLVII, Nos. 2 and 3, 1906.)

## XXXV. A STUDY OF THE SUBSTANCES WHICH ACTIVATE COBRA VENOM.<sup>1</sup>

BY

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### I. Concerning the Activation of Cobra Venom by Means of Complements.

IN a previous study<sup>2</sup> one of us has shown that cobra venom is activated not only by certain active sera but also by lecithin and certain complement-like substances of the red blood-cells called "endocomplements." This, of course, harmonizes with the amboceptor nature of the poison which had been demonstrated by Flexner and Noguchi.<sup>3</sup> In view of the wide distribution of lecithin in the organs and tissues it seemed advisable to penetrate deeper into the mechanism of cobra-venom hæmolysis, especially in order to determine if the assumption of complements and endocomplements is not superfluous and the presence of lecithin in the red blood-cells and serum sufficient to explain the complement action. It is true that certain sera which activate cobra venom lose this property when they are heated to 56° C. for half an hour, and the endocomplements produced by dissolving the red blood-cells in water are inactivated by heating to 62° C. Considering the great ease with which lecithin combines with albuminous bodies, etc., it was possible that the thermolability of the activating factors was simulated by a combination of the lecithin with other substances. An important fact which speaks strongly against this view, however, is one first brought

<sup>1</sup> Reprint from the Berlin klin. Wochensh. 1903, Nos. 2 and 4.

<sup>2</sup> P. Kyes. See page 291.

<sup>3</sup> Flexner and Noguchi, Snake Venom in Relation to Hæmolysis, Bacteriolysis, and Toxicity, Journ. of Exp. Medicine, Vol. VI, No. 3, 1902.

out by Calmette,<sup>1</sup> namely, that almost all sera after being heated to 65° C. and higher usually show even an increased activating power. This we could explain only by ascribing it to the lecithin set free through heating (Kyes, l. c.). It thus appeared that heating was more likely to effect a splitting off than a combination of the lecithin.

Our further studies have shown, however, that this view is not correct in all cases.<sup>2</sup>

To begin, we examined the complementing properties of serum, choosing for our analysis the combination ox blood + cobra venom + guinea-pig serum. The activating property of the fresh guinea-pig serum was destroyed by half an hour's heating to 56° C., and hence was apparently not due to the presence of lecithin, but to some other complement-like substance. Subsequent investigations have confirmed us in this opinion. The general course of the hæmolysis through snake venom is markedly different when lecithin or serum is used for complementing. Lecithin effects rapid solution; with large amounts of cobra venom this is almost instantaneous. When serum is used as complement a longer or shorter period of incubation is observed, such as we are accustomed to see with the hæmolytic sera. Furthermore, hæmolysis with cobra venom + lecithin occurs even at 0° C., whereas the action of cobra venom + serum as complement requires a greater degree of heat.

That the activating substance of the serum belongs to the class of complements was further demonstrated by the fact that it was destroyed by digestion with papain. Following the method of Ehrlich and Sachs,<sup>3</sup> in order to digest the complement, 5 cc. guinea-pig serum were mixed with 1 cc. 10% solution of papain, digested for 1½ hours and then centrifuged. The decanted fluid was used to activate the cobra venom. Table I shows that this property was almost completely lost.

The serum treated with papain had thus almost completely lost its activating property, whereas a solution of lecithin similarly treated preserved its activating property unchanged. (See Table II.)

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<sup>1</sup> Calmette, Sur l'action hémolytique du venin de cobra, *Compt. rend. de l'Académie des Sciences*, T. 134, No. 24, 1902.

<sup>2</sup> We are much indebted to Drs. Lamb and Greig for cobra venom kindly placed at our disposal.

<sup>3</sup> Ehrlich and Sachs, *The Plurality of Complements in Serum*, *Berl. klin. Wochenschr.* 1902, Nos. 14 and 15.

TABLE I

Amount of Serum.  cc.	1 cc 5% Ox Blood + 0.02 cc. 1% Cobra Venom + Guinea pig Serum.	
	(a) Normal.	(b) After Previous Treatment with Papain.
	Amount of Hæmolysis.	
0.5	complete	almost 0
0.35	"	" 0
0.25	almost complete	" 0
0.15	strong	" 0
0.1	"	0
0.075	moderate	0

TABLE II.

Amount of Lecithin Solution.  cc.	1 cc 5% Ox Blood + 0.02 cc 1% Cobra Venom + 0.025% Lecithin.	
	(a) Native	(b) After Previous Treatment with Papain.
	Amount of Hæmolysis	
0.25	complete	complete
0.15	"	"
0.1	"	"
0.075	trace	trace
0.05	0	0

In like manner the complementing property of the serum is destroyed by appropriate digestion with hydrochloric acid and with soda lye, in which again the serum differs from lecithin.

We felt that the discovery of agents which would exert an inhibiting effect in the hæmolytic action of only one of the two factors (either on that of the serum or of the lecithin) would be most valuable for a positive differentiation of serum complement and lecithin. We therefore next immunized rabbits and chickens with guinea-pig serum, seeking in that way to produce anticomplements. By the natural production of an antibody we could thus prove the complement nature of the serum activator. These experiments, however, encountered certain difficulties, for, as we have already mentioned

normal sera exert a considerable inhibition on cobra venom hæmolysis when serum is used as complement, and to a still greater degree when lecithin is used. We observed no essential increase in the protective action in the animals treated with guinea-pig serum. We therefore next sought to distinguish anticomplement and antilecithin action in normal serum.

For this purpose guinea-pig serum itself seemed best suited; inactivated by half an hour's heating to 56° C. it exerts a marked inhibitory action on cobra venom+lecithin hæmolysis. This fact by itself, however, in no way argues against the identity of lecithin and the complementing substance of active guinea-pig serum. One could assume, for instance, that, on heating, a substance is formed which is capable of combining with the lecithin. In that case if an excess of the substance were formed, this would be capable of combining with lecithin subsequently added. This would explain the apparently paradoxical phenomenon that the same serum when fresh exhibits activating properties, but when heated to 56° C. is able to bind lecithin.

We therefore investigated the property of active fresh guinea-pig serum to inhibit the action of lecithin and hoped that this property would still be manifested in dilutions in which the serum was no longer able to exert any activating influence on cobra venom. As a matter of fact we succeeded in proving that guinea-pig serum still exerts an inhibiting influence on the lecithin, even in very small amounts which no longer activate. This is shown by the example in Table III.

TABLE III.

1 cc. 5% Ox BLOOD + 0.001 cc. 1% COBRA VENOM + 0.075 cc. 0.025% LECITHIN.

Amounts of Guinea pig Serum Added. cc.	Hæmolysis.
0.5	complete
0.25	strong
0.1	trace
0.05	0
0.025	0
0.01	trace
0	complete

The lecithin and guinea-pig serum are digested for half an hour previous to adding the ox blood and cobra venom.



Under these circumstances, of course, we can no longer regard the activating factor of guinea-pig serum and lecithin as being identical. If lecithin and serum complement were identical, the antilecithin should act also against the serum complement. In guinea-pig serum, however, as is shown by its activating power, an excess over any such inhibiting substances is surely present and this excess, of course, persists even in quantities of the serum so small as no longer to lead to hæmolysis. A serum protection can therefore be exerted only against substances which are different from the activating substance of the serum.

Further confirmation of this difference was afforded by the fact that we succeeded in demonstrating the existence of antilecithin and anticomplement components in normal rabbit serum inactivated by heating to 56° C. By the addition of lecithin we completely neutralized the components which inhibit lecithin.<sup>1</sup> In fact we added so much that there was a slight excess of free lecithin. Although this mixture in large quantities *was itself activating, in smaller quantities it was able to markedly inhibit hæmolysis with cobra venom + guinea-pig serum.* The anticomplement component of the rabbit serum had been unaffected by the addition of lecithin, as can be seen from the following experiment:

20 cc. rabbit serum are mixed with 180 cc. absolute alcohol, the resulting precipitate rapidly filtered, pressed out, and dissolved in 20 cc. salt solution. This solution protects against cobra venom hæmolysis not only with lecithin activation but also with that of guinea-pig serum.

4 cc. of the inhibiting solution are digested for three-quarters of an hour with 2 cc. of a 0.17% lecithin solution. In large amounts this mixture, through an excess of lecithin, activates cobra venom; in small amounts it inhibits the activation with guinea-pig serum. (See Table IV.)

Besides this we have discovered that cholesterin markedly inhibits, or even entirely prevents, the cobra-venom hæmolysis brought about by lecithin. We shall return to this point later. *In contrast to the behavior of the lecithin we find that the serum complement is practically unaffected by cholesterin,* for only a very slight inhibition is observed even with large amounts of cholesterin, a phenomenon which may be due to absorption. Such an experiment is reproduced in Table V. The solution of cholesterin

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<sup>1</sup> In order to exclude the activating action of rabbit serum, which is due to available lecithin, it is necessary to work with the alcoholic precipitate obtained from rabbit serum. This contains the inhibiting substances.

was made by mixing 1 cc. of a hot, saturated solution of cholesterol in methyl alcohol with 9 cc. hot 0.85% salt solution. This homogeneous suspension of cholesterol served as stock solution for the experiments.

TABLE IV

0.25 CC. GUINEA-PIG SERUM AND THE INHIBITING SOLUTION ARE DIGESTED AT 37° C. FOR THREE-QUARTERS OF AN HOUR. THEREUPON THE OX BLOOD+0.01 1% COBRA VENOM ARE ADDED.

Amounts of the Inhibiting Solution. cc.	Hæmolysis in the Presence of	
	A. Native Solution of the Precipitate.	B. Solution of the Precipitate+ Lecithin.
1.0	faint trace	complete
0.5	trace	almost complete
0.25	little	moderate
0.15	"	little
0.1	moderate	moderate
0.05	"	"
0.025	strong	strong
0.01	"	almost complete
0	complete	complete

TABLE V.

Cholesterolin Solution. cc.	Ox Blood+0.01 cc. 1% Cobra Venom Activated with the Complete Solvent Dose of	
	(a) Guinea-pig Serum.	(b) Lecithin.
0.5	moderate	0
0.25	"	0
0.1	"	0
0.05	strong	0
0.025	complete	0
0.01	"	0
0.005	"	0
0.0025	"	complete

These various experiments lead us to believe that *serum complement* and *lecithin* are two entirely distinct substances. On the other hand the complementing property of the serum for cobra venom corresponds so well with the other complement functions of the sera that no reason at present exists for undertaking a separation. In conformity with this correspondence we find that the activating

substance is absorbed by yeast. In the same connection we may perhaps mention that when fresh guinea-pig serum is shaken with ether it loses not only the other complementing functions but also that for cobra poison. If guinea-pig serum which has been heated to 100° C. (and which therefore owes its activating property to the lecithin liberated through heat) is treated with ether in exactly the same manner, the complementing function remains unchanged.

## II. The Lecithin Content of the Stromata and the Activation of Cobra Venom Dependent Thereon.

In the investigation of the substances in the red blood-cell termed endocomplements we were at first led into error by the employment of just this method of differentiation dependent on the destructibility of complements by means of ether.

For these experiments we used the combination ox blood + cobra venom + solution of guinea-pig blood. The latter was obtained by dissolving sedimented guinea-pig blood in distilled water. The solution was made up to three times the original volume of blood, whereupon NaCl was added until the solution contained 0.85%. If such a solution is shaken with highly purified ether (1 volume blood solution + 10 volumes ether) and a sample of the solution (separated by means of a separating-funnel) is tested it will be found that this has lost its power to activate cobra venom. The ethereal residue taken up in salt solution also exhibited no complementing properties, so that it appeared as though the substance termed "endocomplement" was destroyed by the ether just as were the serum complements. This, however, is not the case. When the blood solution separates after shaking with ether an emulsified stratum is formed between ether and blood solution. On testing that part of the blood solution which contains this intermediary stratum the entire quantity of the activating substance is recovered. (See Table VI.)

*This shows, therefore, that the activating substance had not been destroyed, but that it had escaped our observation, owing to the peculiar behavior of the emulsified stratum.* It is well known that such emulsions take up minute solid particles most readily, and it was natural therefore to assume that the activator contained in the blood-cells is connected with their stromata. In laky blood-solutions the stromata are present in a swollen state; hence we sought to separate the stromata from the rest of the hæmoglobin solution. With guinea-

pig blood solutions this is very simple, for on strongly centrifuging the solution used for complementing, especially if some salt is added, the stromata settle out very well. By removing the supernatant hæmoglobin solution and perhaps once more washing the sediment, the stromata are readily isolated. This suspension of blood stromata, made up to the original volume with salt solution, showed itself just as capable of activating cobra venom as was the original blood solution, whereas the decanted fluid was entirely inert. *The activating substance of the blood solution is present therefore not in solution but as a constituent of the stroma of the blood-cells.* (See Table VII.)

TABLE VI.

Amounts of the Blood Solution.  cc.	Ox Blood + 0.01 cc. 1% Cobra Venom + Blood Solution.		
	(a) Native.	(b) After Shaking with Ether.	
		I Lower Half of the Blood Solution.	II. Upper Half of the Blood Solution (to gether with the emulsi- fied stratum).
1.0	complete	0	complete
0.5	"	0	"
0.25	"	0	"
0.10	"	0	"
0.05	0	0	"
0.025	0	0	faint trace
0.01	0	0	0

TABLE VII.

Amounts of a, b, and c. cc.	Ox Blood + 0.01 cc. 1% Cobra Venom +		
	(a) Guinea-pig Blood Solution.	(b) Suspension of Blood-cell Stromata	(c) Decanted Por- tion.
1.0	complete	complete	0
0.5	"	"	0
0.25	"	"	0
0.15	"	"	0
0.1	little	trace	0
0.05	0	0	0

This threw some light on the inactivation of the blood solution at 62° C., a fact which made the complement character of the activating substance seem exceedingly probable. In contrast to the native blood solution we find that the suspension of stromata remains unchanged when heated to 62° C.

The activating substance itself is therefore thermostable. If, however, the decanted hæmoglobin solution is again added to the stromata and this mixture heated to 62° C. inactivation will again ensue. (See Table VIII.)

TABLE VIII.

Amounts of a, b, and c. cc.	Ox Blood + 0.01 cc. 1% Cobra Venom +		
	(a) Guinea-pig Blood Stromata Suspension.	(b) The Suspension Heated to 62° C.	(c) The Suspension + Decanted Fluid (Hæmo- globin) Heated to 62° C.
1.0	complete	complete	0
0.5	"	"	0
0.25	"	"	0
0.15	"	strong	0
0.1	trace	trace	0
0.025	0	0	0

From this it appears that the inactivation of the native blood depends on this: that on heating to 62° C. the active substance combines with the hæmoglobin in such fashion that it is no longer able to combine with the cobra amboceptor. Hence in view of the closeness with which lecithin combines with albuminous substances, we believe that the activating property of dissolved blood-cells which we previously described as an "endocomplement action" is solely due to the presence of lecithin or lecithin-like substances in the stroma.<sup>1</sup>

We have convinced ourselves of the correctness of this assumption also by the fact that lecithin is bound by crystallized horse hæmoglobin by heating for half an hour to 62° C.<sup>2</sup> An experiment of this kind is reproduced in Table IX.

A solution of hæmoglobin heated for half an hour to 62° C. is also able to inhibit the activating property of lecithin when digested with it for half an hour at 37° C.

The lecithin character of the activating substance present in the blood-cells is confirmed by a number of other observations which agree with the analogous character of cobra-venom hæmolysis on the

We were able to completely extract the activating substance from the stromata suspensions by means of alcohol. Besides this, in activating with stromata in the presence of excess of cobra venom, one observes an inhibition of hæmolysis due to the deflection of the lecithin.

We are much indebted to Prof. Hufner of Tübingen for this hæmoglobin.

addition of lecithin and of blood solution. These characteristics are as follows:

1. The hæmolytic activity at 0°.
2. The comparatively rapid course of hæmolysis.
3. The marked inhibitory action of cholesterin. (See Table X.)

TABLE IX.

Amounts of the Hæmoglobin-Lecithin Solution. cc.	Ox Blood + 0.01 cc. 1% Cobra Venom + Hæmoglobin-Lecithin Solution.*	
	(a) Native.	(b) Heated for One-half Hour to 62° C.
1.0	complete	0
0.75	"	0
0.5	"	0
0.35	little	0
0.25	trace	0
0.15	0	0

\* 5 cc. hæmoglobin × 5 cc. 0.0125% lecithin solution.

TABLE X.

Amounts of the Cholesterin Solution. cc.	1 cc 5% Ox Blood + 0.01 cc. 1% Cobra Venom +	
	(a) 0.25 cc. Solution of Guinea-pig Blood.†	(b) 0.25 cc. 0.01% Lecithin.†
0.025	0	0
0.01	trace	0
0.005	moderate	0
0.0025	complete	complete

† = complete solvent dose.

It will be remembered that in these three points guinea-pig serum exhibited exactly the opposite behavior, a fact which led us to ascribe its activating power to true complements.

We have therefore come to the conclusion that solution by means of blood solutions is only a property of the lecithin contained in the blood-cell stroma, and is not due to true complements. We know that according to Ehrlich's<sup>1</sup> conception the stromata of the red blood-cells are to be looked upon as living protoplasm. In this

<sup>1</sup> Ehrlich, Zur Physiologie und Pathologie der Blutscheiben, Charité Annalen, Vol. X, 1885.

respect the demonstration of lecithin in the stroma would appear to be of special interest, for just this substance is regarded as particularly important for the functions of the protoplasm.<sup>1</sup>

A further problem, to be sure, is whether this lecithin exists free in the red blood-cells. We have a number of reasons for believing that this is not the case. It was first shown that in yolk of egg only a small part of the lecithin can be shaken out with ether, whereas by extracting with alcohol the entire amount can be obtained.<sup>2</sup> The reason for this is that the greater part of the lecithin is conjugated with the vitellin of the yolk. This combination can be obtained as a globulin-like body which is soluble in salt solution and precipitates on dialyzing.<sup>3</sup>

The lecithin is obtained free, however, only after extraction with alcohol, by which the vitellin also changes and becomes insoluble in salt solutions. In demonstrating the presence of lecithin by means of cobra venom we too have observed that the serum and the red blood-cells yield no lecithin to ether, or if they do it is only in faint traces. On the other hand, the active power of the alcoholic extracts at once led to the recognition of the presence of lecithin.

From this point of view some of our earlier observations can easily be explained. We stated that solutions of certain species of blood-cells were strongly activating, while others showed this property to a far less degree or not at all. The alcoholic extracts of all species of blood, however, contain nearly the same amount of lecithin (demonstrated by the activation of the cobra venom). This apparent contradiction is readily explained by the fact that in the different species of blood the lecithin is conjugated with different substances of the stromata and, furthermore, that the firmness of this combination varies extensively. Thus in goat blood the union is so firm that the avidity of the cobra venom does not suffice to separate the two components; the consequence is that there is no activation with a solution of goat blood. On the other hand, in

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<sup>1</sup> It has long been known that lecithin is a constant constituent of the red blood-cells; for many species of blood-cells this content has even been worked out quantitatively. Nothing, however, was thus far known concerning the localization of the lecithin.

<sup>2</sup> See Hoppe-Seyler's *Handbuch der physiologisch- und pathologisch-chemischen Analyse*, Seventh Edition, edited by H. Thierfelder, Berlin, 1903, page 157.

<sup>3</sup> *Ibid.*, page 369.

guinea-pig blood, for example, the lecithin is so loosely combined that this blood can be used for activation. Hence, in speaking of the lecithin action of animal tissues or juices, we refer only to the lecithin which is *free* (available) in the sense just described; part or all of the lecithin present may escape detection by means of the activation of cobra venom.

The fact that relatively slight alterations can cause the combination of lecithin to be either looser or firmer may be of some interest in another direction. We have seen that the lecithin of many species of sera becomes free only at 65° C., while the hæmoglobin, on the other hand, anchors the lecithin at 62° C. It is possible that during life slight variations in the physical and chemical properties of the tissues (variations which have heretofore been undetected) play an important rôle in the sense that they properly regulate the exchange and transportation of the lecithin so important for the vital functions. Dieudonné's<sup>1</sup> researches show that the albuminous bodies with which the lecithin is combined (principally in the form of lecithalbumin) are demonstrably modified, even at temperatures still quite distant from their coagulation point. This author showed that *B. coli*, for example, when inoculated into a serum lactose solution causes a distinct precipitation even at 45° C., while this does not occur at 37° C. In the case of serum albumin, therefore, the temperature at which this modification takes place is very near the temperature which occurs in the living organism under pathological conditions. In view of this and of the evident dependence of the physiological behavior of the lecithin on the integrity of the albumin molecule, one is tempted to see a causal relationship between febrile processes and disturbances in lecithin metabolism.

### III. The Inhibitory Action of Cholesterin.

The marked inhibitory action which many sera exert on hæmolysis with cobra venom and lecithin was described some time ago (Kyes, l. c.) and the opinion then expressed that this protective action of the serum was probably not due to a single substance but was the resultant of several factors. Evidently we are here dealing with certain relations which exist between serum constituents and the lecithin, making

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<sup>1</sup> Dieudonné, Über das Verhalten des *Bact coli* zu nativem u. denaturiertem Eiweiss, Hyg. Rundsch 1902, No. 18.



it impossible to demonstrate the existence of the latter by means of cobra-venom hæmolysis.<sup>1</sup>

Having thus learned that cholesterin exerts a marked inhibiting effect on the action of lecithin we shall probably not err if we assume that part of the serum protection is due to the cholesterin present in the sera. One thing which agrees perfectly with this assumption is the fact that often this protective action is still present after heating the serum to 100° C.

The marked inhibition of hæmolysis on the addition of cholesterin, an inhibition which applies also to the hæmolysis produced by lecithin alone when in large quantities, points to an interesting antagonism between lecithin and cholesterin, to which a few words may be devoted.<sup>2</sup> In this case the cholesterin probably has a relation to lecithin which is similar to that of saponin in Ransom's well-known experiments.<sup>3</sup> In both cases we seem to be dealing with the effect of a kind of solvent affinity between cholesterin on the one hand and lecithin and saponin on the other, by means of which affinity the presence of cholesterin *within* the blood-cells gives rise to toxic action, and *outside* of the erythrocytes exerts a protective action. It is possible that the protection observed by us in hæmolytic test-tube experiments with cholesterin is in some way connected with the protective action of cholesterin against snake venom in the animal body described by Phisalix.<sup>4</sup> Another fact may be mentioned in this connection, namely, that the hæmolysis of washed

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<sup>1</sup>On the other hand the specific protection exerted by Calmette's snake-venom immune serum is not an antilecithin effect, but, as was to be expected, one depending on the action of the antibody produced by immunization (anti-amboceptors) on the amboceptors of snake venom. When varying amounts of lecithin were employed the protective action of Calmette's serum remained constant, always neutralizing the same amount of cobra venom.

<sup>2</sup>We may add that, like Noguchi (The Antihæmolytic Action of Blood Sera, Milk, and Cholesterin upon Agaricin, Saponin, and Tetanolysin, etc., Univ. of Penna. Med. Bulletin, Vol. XV, No. 9, 1902), we observed a very marked cholesterin protection against the action of tetanolysin. (0.00025 cc. of our stock solution, which certainly contains not more than 1% cholesterin, protects against the complete solvent dose of tetanolysin (0.05 cc.)) On the other hand, cholesterin is absolutely without effect on the hæmolyses due to staphytolysin and arachnolysin. In connection with this we might mention the fact so interesting biologically, that even so indifferent a substance as neutral olive-oil dissolves the red blood-cells. This hæmolysis is likewise inhibited by cholesterin.

<sup>3</sup>Ransom, Saponin und sein Gegengift, Deut. med. Wochensh. 1901.

<sup>4</sup>Phisalix, Compt. rend. de la Soc. de Biologie, 1897.

guinea-pig blood-cells, in themselves susceptible to cobra venom alone, is also inhibited by cholesterol. To be sure, rather large quantities of the latter are required, but in view of the lecithin character of the substances which functionate as endoactivators, this is to be expected. (See Table XI.)

TABLE XI.

Amounts of the Cholesterol Solu- tion, cc.	1 cc 5% Guinea-pig Blood $\times$ 0.0025 cc. 1% Cobra Venom.
1.0	0
0.5	0
0.25	little
0.1	marked
0.05	almost complete
0.035	complete

On the other hand, as already remarked, cholesterol exerts little or no protection against cobra-venom hæmolysis when serum complement is used for activation. This agrees entirely with the negative findings on the protective action of cholesterol recently reported by Flexner and Noguchi in an interesting paper on the amboceptor, toxoids, and separate constituents of snake venom.<sup>1</sup>

The apparent deviations are probably to be explained merely by the different conditions of the experiments, for, as it appears to us, these authors made their experiments only on unwashed blood-cells or by the addition of serum. In both cases, however, one is dealing with an activation with complement, against which we also failed to detect any marked protection with cholesterol.

#### IV. The Quantitative Relations Existing Between Cobra Venom and Lecithin.

So far as the mechanism of cobra-venom-lecithin hæmolysis is concerned, we assume that the lecithin acts after the manner of complements, being anchored by certain definite groups of the poison molecule. This has previously been described by Kyes, *l. c.*

Cobra venom and lecithin accordingly combine just like amboceptor and complement in serum hæmolysins, and it was therefore to be expected that the quantitative relations which exist be-

<sup>1</sup> Flexner and Noguchi, The Constitution of Snake Venom and Snake Sera, Univ of Penna. Med. Bulletin, Vol. XV, No. 9, 1902.

tween amboceptor and complement would be very similar in this case. In our studies in hæmolysis due to cobra-venom-lecithin we have therefore been able to observe the same mutual dependence between amount of amboceptor present and the complement required which the researches of von Dungern,<sup>1</sup> Gruber<sup>2</sup> and Morgenroth and Sachs<sup>3</sup> showed to exist in their experiments. The relation between these amounts is such that when large amounts of amboceptor are present, smaller doses of complement suffice for hæmolysis.

To be sure, when an inordinately large amount of cobra venom is added the amount of lecithin required for complete solution is also larger, as has already been mentioned by Kyes. This is evidently explained by assuming that when the amount of amboceptor is excessive the distribution of the lecithin is such that part of the amboceptor loaded with lecithin is deflected and does not come into action. If, however, the amount of cobra venom is decreased, results will be obtained which, within wide limits, agree with those observed by Morgenroth and Sachs (l. c.) with serum hæmolysins. *The more cobra venom one adds the less lecithin will be needed to effect complete hæmolysis*, and, conversely, in adding larger amounts of lecithin the minimal complete solvent dose of the cobra venom is constantly decreased. This is well shown by Table XII.

TABLE XII.

A. 1 cc. 5% Ox Blood.		B. 1 cc. 5% Ox Blood.	
Amounts of the 1% Solution of Cobra Venom.	The Amount of Lecithin Solution (0.025%) Necessary for Complete Solution.	Amounts of the 0.025% Lecithin Solution.	The Amount of Cobra Venom (1%) Necessary to Effect Complete Solution
0.01	0.035	0.3	0.00001
0.001	0.05	—	—
0.00025	0.075	0.06	0.0001
0.0001	0.1	—	—
0.00001	0.5	0.03	0.005

From these experiments we see that the quantitative relations which exist between cobra venom and lecithin furnish an additional

<sup>1</sup> von Dungern, page 36.

<sup>2</sup> Gruber, Wiener klin. Wochenschr. 1902, No. 15.

<sup>3</sup> Morgenroth and Sachs, pages 233 and 250.

argument for the view that cobra venom and lecithin behave like amboceptor and complement.

#### V. The Susceptibility of the Red Blood-cells.

These observations show that in comparing the susceptibility of the various species of blood to cobra venom the limit of activity of the venom must be determined with the optimum quantity of lecithin. The values thus obtained may be regarded, so to speak, as the "absolute susceptibility" of the blood-cells. In Table XIII the minimal complete solvent dose is determined for several species of blood on the addition of an abundant quantity of lecithin (0.2 cc. of a 0.025% lecithin solution).

TABLE XIII.

Species of Blood (1 cc. of a 5% Suspension).	Amount of Lecithin. cc.	Complete Solvent Dose of Cobra Venom. Gram.
Guinea-pig....	0.2 cc. of a 0.025% sol.	0.00000005
Ox.....	"	0.0000001
Rabbit.....	"	0.00000025
Man.....	"	0.0000005
Goat.....	"	0.000001

If we compare these values with the susceptibility of the various blood-cells with cobra venom alone (see Table XIV) we shall see that when the latter is used the amount of venom necessary for complete hæmolysis is many times greater than when a sufficient amount of lecithin is added. Thus the absolute susceptibility of guinea-pig blood against cobra venom+lecithin is 500 times greater than that obtained without the addition of lecithin.

This shows also that although guinea-pig blood heads the list in either case there are marked deviations, so far as the other bloods are concerned, from the results obtained on the addition of lecithin. Ox blood, for example, which is not at all susceptible when lecithin is lacking, is more susceptible than either rabbit or human blood when lecithin is present. Yet the two latter species of blood are dissolved even without the addition of lecithin.

We thought it would be especially interesting to study the susceptibility of human blood-cells to cobra venom in various diseases. In the few cases thus far observed (several healthy persons, two cases

of diabetes, one of pneumonia, and one typhoid) we were unable to discover any essential difference in susceptibility.<sup>1</sup>

TABLE XIV.

## SUSCEPTIBILITY OF VARIOUS SPECIES OF BLOOD TO COBRA VENOM ALONE

Species of Blood (1 cc. 5% Suspension).	Amount of Cobra Venom Required for Complete Hemolysis.
Frog.....	0.00001
Dog.....	0.000025
Guinea-pig.....	0.000025
Man.....	0.00005
Rat.....	0.00025
Pig.....	0.00025
Mouse.....	0.00025
Goose.....	0.0005
Rabbit.....	0.001
Horse.....	0.001
Ox, sheep, goat.....	not susceptible

As a result of our extensive researches we must continue to uphold the view that blood species are clearly divisible into those directly susceptible to cobra venom alone and those not susceptible under those conditions. This follows also from the above table. In this respect our observations are at variance with the recent statements of such excellent workers as Flexner and Noguchi. It may be well therefore once more to point out a few possibilities by which this difference can be explained. Flexner and Noguchi observed that, in general, after copious washing, the blood-cells were not dissolved by cobra venom, or at least were only partially dissolved. In spite of repeated washing of the blood we were unable to discover any decrease in susceptibility.

If Flexner and Noguchi insist on such a thorough washing (6-10 times) it appears to us that it can no longer be a question of removing the serum complements. The small quantities of serum which are contained in the 0.05 cc. blood employed in each tube in the test-tube experiment (1 cc. of a 5% suspension) are entirely too small, according to our experience, to exert a demonstrable complement

<sup>1</sup> It is possible that investigations in other diseases will lead to positive results. We are not in a position to apply our observations to more extensive clinical material, but shall be glad to supply cobra venom for this purpose to any one applying for the same.

action after one or two washings. We are therefore more inclined to assume that the insusceptibility observed by Flexner and Noguchi is due to a washing out of the activating substances present in the blood-cell. One of us has already reported such extraction phenomena (Kyes, l. c.); we have, however, been unable to repeat the experiments. It is possible, as has already been stated, that the divergent results are due to minute differences in the experiment, differences which for the present at least cannot be analyzed. It is also possible that a certain degree of racial divergence in the blood-cells of animals of the same species used by Flexner and Noguchi and by us gives rise to what at present is an inexplicable difference. In the blood-cells employed by us the activating substances could not readily be washed out. This is shown by the fact that the activating substances are so firmly bound to the protoplasm that they are not separated even in preparing the stromata.

Attention is also called to the antagonism which is so often observed between blood-cells and their own serum. This has already been pointed out by Kyes. Thus rabbit blood-cells are dissolved by cobra venom, and this action is intensified by the addition of rabbit blood-cells which have been made laky. In spite of this, however, the active serum of the same rabbit inhibits cobra-venom hæmolysis (see Table XV). In this case, therefore, adherent traces of serum cannot possibly effect autoactivation of the rabbit blood-cells.

TABLE XV.

Amounts of the 1% Cobra Venom.  cc.	1 cc. 5% Rabbit Blood +		
	Cobra Venom Alone.	Cobra Venom + 0.05 cc. Rabbit Serum.	Cobra Venom + 0.05 cc. Rabbit-blood Solu- tion (†).
0.1	complete	0	complete
0.05	almost 0	0	"
0.025	0	0	"
0.01	0	0	"
0.005	0	0	"
0.0025	0	0	"

There is another point of considerable interest in connection with these questions, one very important for the technique. The susceptibility of the washed blood-cells can readily be overlooked in

many cases owing to the occurrence of a marked inhibition of hæmolysis due to the presence of an excessive amount of cobra venom.

Kyes (l. c.) has already discussed in detail the fact that in hæmolysis with cobra venom alone a phenomenon can occur which is analogous to the deflection of complement described by M. Neisser and Wechsberg.<sup>1</sup> In rabbit blood we have observed extensive individual differences so far as this deflecting phenomenon is concerned. We have often found animals whose blood-cells remained undissolved in the presence of even a very slight excess of cobra venom, so that it was necessary to have just the right amount of venom in order to effect hæmolysis. Table XVI shows several examples of this.

TABLE XVI.

Amounts of 1% Cobra Venom	1 cc 5% Rabbit Blood.			
	Rabbit I.	Rabbit II.	Rabbit III.	Rabbit IV.
1.0	0	—	—	—
0.5	faint trace	—	—	—
0.25	little	—	—	—
0.1	complete	0	trace	complete
0.075	almost 0	complete	—	"
0.05	0	moderate	marked	"
0.025	0	0	complete	"
0.01	0	0	trace	"
0.005	0	0	0	strong
0.0025	0	0	0	trace
0.001	0	0	0	almost 0
0.0005	0	0	0	0

The marked deflection which is observed in the blood of rabbits I, II, III is evidently caused by a relatively slight amount of activating substances present in and at the disposal of the red blood-cells. On the other hand the different behavior of other bloods, as in rabbit IV, shows how the amount of free lecithin contained in the blood-cells can vary from case to case. It might pay to examine the blood of different rabbits for this purpose.

<sup>1</sup> See page 120.

## VI. A Few Chemical Considerations.

Finally, we should like briefly to discuss some of our experiences with the power possessed by certain other substances to activate cobra venom. In view of its content of lecithin, it will not surprise us to know that bile activates cobra venom. It may be interesting, however, to learn that goat milk acquires activating properties only when it has previously been heated to 100° C. This behavior corresponds entirely to that of certain species of sera whose lecithin does not become available until after they have been heated to 65-100°. Among chemical substances we have found a number of fatty acids and their soaps, chloroform, and olive oil able to activate to a certain degree. All these substances by themselves, however, dissolve the blood-cells to a greater or less degree<sup>1</sup> and the increase of this action is so slight that it is doubtful whether we can here speak of pure activating phenomena.<sup>2</sup>

According to our experiences only one more substance, namely, the lecithin-like cephalin, possesses marked activating properties. (Cerebrin does not possess them.) For this cephalin we are indebted to Waldemar Koch of Chicago, who made it from sheep's brain. According to him, it is a dioxystearylmonomethyl lecithin.<sup>3</sup> The cephalin (which is insoluble in alcohol) and the lecithin (which is soluble in alcohol), both made by Koch from sheep's brains, furthermore two other preparations of lecithin (one from Riedel in Berlin, the other kindly placed at our disposal by Dr. Bergell), all these manifested a hæmolytic action (if at all) only in 500-600 times the amount sufficient to activate the cobra venom.

A preparation of lecithin derived from leguminous seeds, for we are indebted to Prof. Schulze of Zurich, showed less difference between activating power and hæmolytic action, but even

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<sup>1</sup> It is possible that the coctostable hæmolysins (soluble in alcohol-ether) of the organ extracts belong in the same class with these substances (see Korschun and Morgenroth, page 267).

<sup>2</sup> It must always be borne in mind that the activating property of these substances may possibly only be an indirect one, the presence of the substance sufficing to make available the lecithin always present in the blood-cells in combination.

<sup>3</sup> W. Koch, Zur Kenntniss des Lecithins, Cephalins und Cerebrins aus Nervensubstanz, *Zeitsch. f. physiol. Chemie*, Vol. 36, Nos 2 and 3, 1903.



ratio was still 1:200. A lecithin obtained from E. behaved similarly. Nevertheless all of these preparations are equal in their activating power. It is hard to say whether the cholin radical or the fatty-acid radical represents the active group of the combination formed by the union of lecithin with the cobra venom. It may be mentioned, however, that neutralized cholin exerts no hæmolytic effect, and that sinapin<sup>1</sup> (an acid ether of cholin), despite the cholin radical which it possesses, exerts no activating power. We are therefore inclined to think that the toxic action is caused by the fatty-acid radical in the molecule. This also agrees with the hæmolytic action of lecithin in neutralized stearyl-glycerophosphoric acid and in neutralized fats and fatty acids. We shall report on further results in this direction at a subsequent period.

In this connection we may be permitted to discuss briefly a few observations. Among these is the fact that hydrochloric acid exerts no destruction or weakening of the cobra venom, but exerts a marked protective action on the same. A venom which completely loses its activity by heating to 100° C. for 10 minutes can be heated for half an hour to 100° C. without losing its hæmolytic property if it contains  $\frac{1}{18}n$  HCl. Not until the acid is heated for two hours to 100° C. is the action complete. Possibly the protection exerted by the acid is due to the basic character of those binding groups of the venom molecule which are here concerned. So far as the action of other agents on the cobra venom is concerned we shall mention that all procedures which prevent the action of the venom in the animal body<sup>2</sup> (an action due mainly to the hæmolytic components of the poison<sup>3</sup>) also destroy the hæmolytic action of the venom. Examples of this are powerful oxidizing substances such as potassium permanganate, chloride of lime, chloride of gold, etc.).

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We are indebted to Geheimrath Schmidt in Marburg.

Especially the detailed and excellent investigations of Calmette, *Annales de l'Institut Pasteur*, T. VIII, 1894.  
and Noguchi, l. c.

*Résumé.*

1. The property of certain sera to activate cobra venom, a property which is lost by heating the sera to 56° C., depends on the presence of complements in the restricted sense.
2. The activating property of blood solutions depends on the lecithin contained in the red blood-cells; this also gives rise to the susceptibility of the blood-cells against cobra venom alone. The lecithin which comes into play is a constituent of the stromata.
3. The fact that blood solutions are inactivated by heating to 62° C. is due to the combination at this temperature of the lecithin with the hæmoglobin; suspensions of blood stromata are not inactivated at this temperature.
4. Cholesterin inhibits to a high degree hæmolysis by means of cobra venom alone, and of cobra-venom-lecithin. When serum complements are used for activation, cholesterin exerts little or no protective action.
5. Cholesterin does not inhibit hæmolysis due to staphylolysin and arachnolysin, but very markedly inhibits that due to tetanolyisin and to olive-oil.
6. The quantitative relations between cobra venom and lecithin correspond to those of amboceptor and complement; the more cobra venom present the less lecithin will be required for hæmolysis, and vice versa. A deflection of lecithin does not occur unless very large amounts of cobra venom are used.
7. Most species of blood are susceptible even to cobra venom alone. The "absolute susceptibility" determined with the optimum addition of lecithin may be many times that obtained without the addition of lecithin.
8. Hydrochloric acid exerts a marked protection on cobra venom against destruction through high temperatures. Potassium permanganate, chloride of lime, chloride of gold, soda lye destroy cobra venom (experiment with blood+lecithin).
9. Bile activates cobra venom; milk (goat) only after it has previously been heated to 100° C.
10. Fatty acid, soaps, chloroform, and neutral fats have a hæmolytic action. The hæmolytic action is somewhat increased on the addition of cobra venom.
11. Lecithin and cephalin, on the other hand, exert a hæmolytic

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action on the ordinary species of blood only, if at all, when 200 or 600 times the amount is used which suffices for activating the cobra venom.

12. In the poisonous combination formed on the union of cobra venom with lecithin the fatty-acid radical may, with a certain degree of probability, be regarded as the active group.

## XXXVI. THE ISOLATION OF SNAKE VENOM LECITHIDS.<sup>1</sup>

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SPECIAL interest attaches to the study of snake venoms because of the analogy which exists between their peculiar character and that of bacterial toxins. All investigators who have worked with this subject have been struck by this analogy, and Phisalix<sup>2</sup> has discussed it in a special monograph. The analogy between snake venoms and bacterial toxins consists, above all, in the fact that neither are crystallizable, that their constitution is unknown, that both are highly virulent specific products of poison-forming cells, and both possess the power to excite the production of antibodies in the organism. This last fact we know from the fundamental researches of Calmette.<sup>3</sup>

A further analogy between snake venoms and the toxins is the fact that the poisonous properties of both are destroyed by heat, and that the non-toxic substance thus formed is able to excite the production of antibodies just as well as the original substance. In other words, in both poisons there is a formation of toxoid. Snake venom has accordingly played an important rôle in the theoretical doctrine of immunity. Martin and Cherry,<sup>4</sup> for example, by their well-known filtration experiment were able to prove that snake venom and specific antitoxin unite to form a new non-poisonous combination. This experiment is based on the principles first formulated by Ehrlich

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<sup>1</sup> Reprint from the *Berliner klin. Wochens.* 1903, Nos. 42, 43.

<sup>2</sup> Phisalix, *Étude comparée des toxines microbiennes et des venins*, *L'Année biologique* I, 1895.

<sup>3</sup> Calmette, *Ann. de l'Institut Pasteur*, No. 5, 1894.

<sup>4</sup> Martin and Cherry, *Proceedings of the Royal Society*, Vol. LXIII, 1898.

in his studies on ricin and antiricin, and the results are entirely similar to Ehrlich's.<sup>1</sup>

Still another important analogy between snake venoms and bacterial poisons consists in their plurality, a fact which has been demonstrated for a number of poisons. In the ordinary well-defined chemical poisons we are accustomed to regard the diverse toxic phenomena as due to the action of one and the same substance on different organs. (In poisoning with corrosive sublimate, for example, the diverse toxic phenomena which are produced in the various organs.) The toxins, however, have to a large extent shown a different behavior, the action on different organs being ascribed to different kinds of poisons, which frequently possess different haptophore groups. The possibility of correctly and sufficiently analyzing these poisons depends in a large measure on Ehrlich's theory of the combination of these poisons. In this way it has been shown that tetanus toxin consists of at least two components, tetanospasmin and tetanolysin,<sup>2</sup> to which, according to Tizzoni, a third poison must be added, one which gives rise to the cachexia.

In snake venom the conditions are entirely similar, the different effects which it produces in the animal body being due to the presence of different poisons with different haptophore groups. The late lamented Myers<sup>3</sup> showed that the hæmolytic property of snake venom is to be separated from its neurotoxic property; and recently Flexner and Noguchi<sup>4</sup> have shown that the œdematous swellings produced by injections of snake venom are due to the presence of a third toxic component acting on the endothelium.

For some years I have closely studied cobra venom, and especially that constituent of the same which causes solution of the red blood-cells. Part of these researches were conducted conjointly with Dr. H. Sachs.<sup>5</sup> I was able to confirm the interesting observation of Flexner and Noguchi<sup>6</sup> that the snake venom, as such, did not act on certain blood-cells, but that hæmolysis occurred only when a second substance is present which acts after the manner of a complement.

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<sup>1</sup> Ehrlich, *Fortschritte der Medizin*, 1897.

<sup>2</sup> Ehrlich in Madsen's paper, *Zeitschrift f. Hygiene*, Vol. XXXII, 1899.

<sup>3</sup> Myers, *Journal of Pathology and Bacteriology*, 1900, VI, 405.

<sup>4</sup> Flexner and Noguchi, *Univ. of Penna. Medical Bulletin*, Vol. XV, No. 9, 1902.

<sup>5</sup> Kyes, see page 291; Kyes and Sachs, see page 443.

<sup>6</sup> Flexner and Noguchi, *Journal of Exp. Medicine*, Vol. VI, No. 3, 1902.

By following up a very important observation made by Calmette,<sup>1</sup> that the complementing action of a serum, in contrast to what is seen with ordinary complements, is still preserved after heating to 62° C., we succeeded in discovering what this complementing agent was, and proved that lecithin was able to activate the cobra venom amboceptor. Especially were we able to show that the divergent behavior of the various species of blood-cells (some of them, ox blood, goat blood, sheep blood, are not dissolved by cobra venom alone, while others, such as guinea-pig blood, rabbit blood, human blood, dog blood, are dissolved under these circumstances) is due exclusively to the lecithin, only those blood-cells being dissolved in which the lecithin is so loosely bound that it is available for the activation of the cobra-venom amboceptor.<sup>2</sup>

An exact study of these activating phenomena by means of lecithin seemed to us to be of the highest importance for one of the fundamental problems of immunity, namely, the mode of action of complements. Every one who has had any large experience with the activation of ordinary hemolytic amboceptors by means of complements, and who compares this activation with that of cobra venom by means of lecithin, will be surprised at the complete similarity of both processes, and will not doubt that essentially the same mechanism must control both. For some years the schools of Bordet and Ehrlich have had a sharp conflict of opinion concerning the explanation of the fundamental facts observed by Ehrlich and Morgenroth, that the amboceptor is anchored by the red blood-cells, thus making the blood-cells susceptible to the action of the complements. For numerous reasons which are given in the earlier studies, Ehrlich's school assumes that

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<sup>1</sup> Calmette, *Compt. rend. de l'Acad. des Sciences*, T. 134, No. 24, 1902.

<sup>2</sup> Some time ago we confirmed the observations of Flexner and Noguchi, that blood-cells unsuspceptible to cobra venom alone can be activated by certain fresh sera, and that this activatibility is then lost on heating the sera to 56° C. In conformity with these authors we assumed that the cobra venom could also be activated by true complements. At present, however, we have become rather skeptical as to the correctness of this explanation. We cannot at once dismiss the assumption that the action is an indirect one, the action of the serum causing the lecithin combination in the red blood-cells to become looser, so that then this substance can exert its activating power on the amboceptor. This finds further support in several observations which we have made on the favoring influence of certain indifferent substances (oils, pure fatty acids) on hemolysis with cobra amboceptor. In these cases the cause of the solution can only be a change in the character of the lecithin combination.

complement and amboceptor unite to form a new poisonous combination, and this is the view which I also take. Bordet, however, even in his latest study<sup>1</sup> assumes that there is no direct affinity between complement (alexin) and amboceptor, but "que la sensibilatrice modifie l'élément de manière à lui faire acquérir le pouvoir de fixer directement l'alexine avec beaucoup d'énergie."

The Frankfurt Institute has furnished a number of important arguments for the direct union of amboceptor of complement. Because of the lability and great number of the complements as well as the impossibility to isolate the active product chemically it was out of the question to furnish direct chemical proof for these views. Nor is there in the present state of scientific knowledge any hope that this problem will be solved in the near future. For this reason we rejoiced in the discovery that in lecithin we had found a substance possessing complement-like properties, and which because of its chemical behavior would serve to settle this dispute.

In other words, it was to be seen whether or not the cobra amboceptor combined directly with the lecithin to form a new hæmolytic combination. If it did not do so, it would help sustain Bordet's view that the union of the cobra venom amboceptor serves only to give the lecithin access to the blood-cell. From the following studies it will be seen that the decision which we had been led to expect as the result of our biological experiments is confirmed by chemical means.

One thing especially argued for the correctness of our conception, namely, the fact that it is possible to inhibit the cobra venom hæmolysis by employing very large amounts of cobra amboceptor. In that case susceptible blood-cells which can be dissolved by a certain definite amount of cobra amboceptor are no longer dissolved if many times this amount is employed. This corresponds to the phenomenon which we observe in certain bactericidal sera, and which according to Neisser and Wechsberg is due to a deflection of complement through an excess of free amboceptor. The result is comprehensible only on the assumption of a direct chemical affinity between amboceptor and complement. For this reason we felt that it would be of the greatest interest to gain a clearer insight through

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<sup>1</sup> Mode d'action et origine des substances actives, des sérums préventifs et des sérums antitoxiques. Rapport présenté par J. Bordet au Congrès de Hygiène et Démographie, 1903.

chemical means into the analogous deflection of complement observed with cobra amboceptor.

### I. Preparation of Cobra Lecithids.

Owing to the tenacious character and the slight solubility of lecithin in water it was, of course, impossible to attempt to effect the desired combination by direct mixture of the aqueous solution of cobra venom with lecithin. On the contrary it was necessary to adopt a common chemical procedure, "shaking out," whereby, through the agency of an appropriate solvent, the lecithin could combine with the cobra venom. After a number of trials we found the best solvent for this purpose to be chloroform.

In our experiments we employed dried cobra venoms which had kindly been placed at our disposal by Dr. Lamb and Dr. Greig of Bombay, and Prof. Calmette of Lille. The lecithin used was the so-called "Lecithol" of Riedel, and later on "Agfa-lecithin" of the Actien-Gesellschaft für Anilin-Fabrication. Both of these proved to be excellent. Special emphasis must be laid on a sufficient purity of the lecithin. For our purposes this is best recognized by testing it against red blood-cells. 0.5 cc. of a 1% solution of the lecithin should not dissolve red blood-cells. If the contrary is the case the lecithin should be purified by precipitating it once or twice with acetone.<sup>1</sup>

Forty cc. of a 1% solution of cobra venom in a 85% salt solution are mixed with 20 cc. of a 20% solution of lecithin in chloroform. The mixture is placed in a bottle holding about 100 cc. and thoroughly shaken for two hours in a shaking apparatus. Thereupon the mixture is centrifuged for three-quarters of an hour in an electric centrifuge making 3600 revolutions per minute. If the procedure has been successful the chloroform layer must then be distinctly separated from the watery portion, only a very slight compact, cloudy, intermediate layer being present. If the lecithin is not sufficiently pure this separation will not take place. The watery portion is separated from the chloroform layer by carefully pipetting off the former. The chloroform layer, usually measuring about

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<sup>1</sup>We were also able to activate cobra amboceptor with a brom-lecithin which Dr. Bergell kindly placed at our disposal. This preparation proved less active than lecithin, but it evidently possesses the power to unite with cobra amboceptor to form a lecithid.



19 cc., is then mixed with five times its volume of chemically pure ether which has been distilled over sodium. A precipitate forms consisting of the desired cobra venom lecithid, while the lecithin remains dissolved in the ether.

Precipitate and fluid are separated by means of the centrifuge, the original volume of ether again added, shaken, and the mixture once more centrifuged. This is repeated at least ten to twenty times in order to remove any adherent lecithin. *The substance thus obtained is the cobra venom lecithid.*

The product can be kept for a long time under ether, apparently undergoing little or no change; or it can be carefully dried, through which, however, it suffers some change, affecting especially its solubility but not its action. The yield of dry substance is quite large, 1 grm. of dry cobra venom yielding about 5 grms. dry lecithid.<sup>1</sup>

After having worked out the best method for obtaining the cobra lecithid it was next necessary to determine by biological means whether the product isolated by us showed itself through its specific action to be the cobra-amboceptor-lecithin combination sought for. That this was actually the case could be proven in two ways, namely:

1. By showing that the extracted watery fluid has lost its hæmolytic property, and
2. By showing that this property is now present in the chloroform-lecithin solutions (see Table I).

So far as the behavior of the aqueous solution is concerned it can actually be shown that the single treatment with chloroform-lecithin removes all but traces of the hæmolytic power, and that a repetition of the procedure suffices to remove all of the hæmolytic agent from the watery solution. Corresponding to this we find that the hæmolytic power of the watery solution has been transferred completely to the chloroform-lecithin portion, a fact which shows that the lecithin has united with the cobra venom (Table I).

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<sup>1</sup> If one has but very little of the primary substance at one's disposal another method of preparing the lecithid can be tried, one which will answer at least for preliminary examinations. 1 cc. of a 4% aqueous solution of cobra venom is mixed with 1 cc. of a 20% solution of lecithin in methyl alcohol, the mixture is kept in an incubator for several hours and frequently shaken; then 10 cc. absolute ethyl alcohol are added and the precipitated albuminoids separated by filtration. On precipitating the clear filtrate with ether, one obtains the lecithid.

TABLE I.

1 cc. 5% Ox BLOOD + 0.2 cc. 0.1% LECITHIN.

cc.	A. Native 0.001% Cobra Venom (Control).	B Cobra Venom Shaken Once with 0.1% Chloroform Lecithin	B Cobra Venom Shaken Out Twice with 1.0% Lecithin- Chloroform.	C Cobra Lecithid from Chloroform- Lecithin by Pre- cipitating with 0.002% Ether <sup>1</sup>
1.0	complete	complete	nil	complete
0.75	"	"	"	"
0.5	"	"	"	"
0.35	"	"	"	"
0.25	"	"	"	"
0.15	"	"	"	"
0.1	almost comp.	"	"	"
0.075	marked	"	—	"
0.05	little	"	—	almost complete
0.035	trace	almost comp.	—	marked
0.025	almost nil	moderate	—	little
0.01	nil	little	—	trace
0.0075	"	trace	—	almost nil
0.005	"	almost nil	—	nil
0.0035	"	nil	—	"
0.0025	"	"	—	"
0.0015	"	"	—	"
Number of solvent doses reckoned on the total original vol- ume of 40 cc. ....	266,000 to 267,000	800	0.0	266,000 to 267,000
Percentage of hæmoly- sins in each solution.	100%	0.003%	0.0%	100%

<sup>1</sup> In comparison to the original aqueous solution of venom.

The assumption that the cobra amboceptor is extracted by chloroform alone is refuted by control tests.

The neurotoxic action of the native poison is entirely absent in the cobra lecithid. Relatively large quantities of the lecithid in aqueous solution can be injected subcutaneously into animals without producing constitutional symptoms. For example, an amount of lecithid which suffices to destroy 200 cc. mouse blood can be injected into mice weighing 15 grms. without causing any further symptoms than infiltration at the site of injection. In like manner rabbits can be injected subcutaneously with 10 cc. of a 1% solution of the lecithid without causing any constitutional symptoms. In this case, however, the local reaction is extensive, the infiltrated area often including a considerable portion of the abdominal surface.

According to this, therefore, the second constituent of cobra venom does not pass into the chloroform-lecithin. In this respect, however, we have been able to demonstrate that the watery portion which has practically been freed from the hæmolytic amboceptor still possesses its toxic properties in animal experiments. (See Table II.) The essential difference between the hæmotoxin and the neurotoxin, first pointed out by Myers, is thus confirmed by direct chemical means.

TABLE II.

COMPARATIVE TEST OF THE NEUROTOXIC ACTION OF A SOLUTION OF COBRA VENOM (a) BEFORE AND (b) AFTER SHAKING THE COBRA AMBOCEPTOR WITH CHLOROFORM-LECITHIN.

0.01% Venom.	(a) Native Venom.	(b) Extracted Venom.
0.5	† after 2 hours	† after 1 hour
0.35	† " 2½ hours	† " 1½ hours
0.25	† " 1¾ "	† " ¾ hour
0.15	† " 2½ "	† " 8 hours
0.12	† " 30-40 hours	† " 30-40 hours
0.1	living	living

## II. The Properties of Cobra Lecithids.

In the description of cobra lecithid we shall do best to keep to the product obtained by the method above described, the last traces of lecithin having been removed from the ethereal precipitate by repeated washing with ether, and the main portion of the ether in turn removed by pressing the precipitate between two folds of filter-paper.

This *primary* product is insoluble in acetone and ether, but soluble in chloroform, in alcohol (cold), and in toluol (on heating). The solutions in chloroform and in alcohol are precipitated by the addition of ether and acetone. When still moist with ether it dissolves very readily in water, a point of some importance. Even if the ether which the product contains is rapidly evaporated by means of a current of air and the product then dissolved in water an absolutely clear, light-yellow solution will be obtained.

These facts show that the primary product is absolutely different from the two substances from which it was derived, cobra amboceptor and lecithin. It differs from lecithin particularly in its insol-

bility in ether and its ready solubility in water; from cobra venom amboceptor in its solubility in the above-mentioned organic solvents, alcohol, chloroform, toluol. Cobra venom does not give up even a trace of cobra amboceptor to these solvents.

It has been found that the watery solution of the primary cobra lecithid obtained from cobra venom and lecithin, as described above, undergoes spontaneous modification which leads to the formation of an insoluble substance. If the watery solution is allowed to stand at room temperature it gradually becomes cloudy, and in the course of a few hours a whitish precipitate is formed. On removing this precipitate, either by filtration or by centrifuge, a precipitate will again form in the clear fluid. The sediment is microcrystalline, white, transparent, and very refractile.

It can easily be shown that this sediment is nothing but a modified form of the lecithid, for after thoroughly washing the precipitate which has been separated by the centrifuge, it will be found that this still exerts its full hemolytic action. In accordance with this, the original solution of the primary product shows a proportionate loss of power. In one experiment which we followed rather closely we found that in course of time about two-thirds of the lecithid had separated out in solid form, while one-third was still left in solution. The *secondary* lecithid produced in this way is, as already stated, almost insoluble in cold water; on the other hand, it is readily soluble in warm water, although it again separates on cooling. This behavior constitutes the chief difference between the primary and the secondary lecithid; the behavior of the two substances toward the above-mentioned organic solvent is identical.

Owing to its character the secondary lecithid is particularly adapted for chemical investigations, and one of the foremost authorities has already commenced work on this substance. Some important results which have already been obtained will be mentioned later on. For the present we shall merely mention that the product gives no biuret reaction even when in concentrated solutions. We are reserving for future study the chemical study of the above lecithids, as well as the investigation of the neurotoxin obtained in purified form by means of the method above described.

The formation of the secondary lecithid also occurs if the ethereal precipitate is dried at incubator temperature. It is then easy to see that such a product has more or less completely lost its solubility in water, especially if it has remained in the thermostat for several days.

In its properties the insoluble portion corresponds entirely with the secondary lecithid precipitate from aqueous solutions. To obtain the secondary lecithid as a pure product, however, the method first mentioned seems preferable, namely, that which starts with the aqueous solution of the primary substance; the result seems to be a lighter-colored product.

It is natural that this lecithid when finished differs in its action in many ways from the cobra amboceptor. It can readily be understood that the cobra lecithid acts on the blood-cells of all the species thus far examined, no matter whether these cells possess available lecithin or not. One fact of considerable interest has been discovered, namely, that the absolute quantity of lecithid necessary for hemolysis is the same for the blood-cells of different species. We found that an amount of lecithid which corresponded to about 0.003 mg. dry cobra venom was able to dissolve 1 cc. of a 5% suspension of blood-cells of different species (guinea-pig, rabbit, man, ox). This quantity, we may add, corresponds to the amount of cobra venom which causes solution of the blood-cells in the ordinary test when a large excess of lecithin is present.

An observation which is also of considerable interest is a comparison of the time necessary for the action of the cobra lecithid and the amboceptor, with and without the addition of lecithin. In our previous papers we pointed out that when cobra venom is allowed to act on susceptible blood-cells, solution occurs after a considerable period of incubation, so that in case a minimal quantity is employed 12 to 18 hours (two hours at 37°, then at 8°) elapse before complete solution is effected. Even if a suitable excess of cobra venom and the most susceptible species of blood are employed, at least 10 to 30 minutes will usually elapse before solution is completed. Similar differences are observed if, as previously described, we allow cobra venom and lecithin to act on unsusceptible blood-cells. In that case, again, with minimal quantities of lecithin and amboceptor 6 to 18 hours are necessary to effect solution; this time is decreased if large excesses are employed, but solution is never instantaneous.

In contrast to this a marked decrease in the period of incubation is observed if the finished lecithid is used, solution being instantaneous on the employment of concentrated solutions. The shortening in the time necessary for solution becomes particularly marked when small doses of the lecithid are used, solution commencing at once

and being completed within 15 to 20 minutes. In other words, the increase in the rapidity of the process is about twenty-fold.

This behavior is significant, for it shows that in this case the period of incubation is due not to a slow action of the anchored toxophore group (lecithin), but exclusively to the slow development of the real toxic agent, the lecithid. The difference in the time of action in the case of small and large doses is in accordance with the well-known law that the reaction (in this case the union of cobra amboceptor and lecithin) proceeds more rapidly in concentrated solutions than in weak ones.

TABLE III.

Amounts of Cholesterin Solution. <sup>1</sup>	1 cc. 5% Ox Blood		
	A. Native Cobra Venom, about 1½ Solvent Doses with the Addi- tion of Lecithin.	B. Primary Cobra Lecithid, about 1½ Solvent Doses.	C. Secondary Cobra Lecithid, about 1½ Solvent Doses
0.1	0	0	0
0.075	0	0	0
0.05	0	0	0
0.035	0	0	0
0.025	0	0	0
0.015	0	almost 0	almost 0
0.01	0	trace	trace
0.0075	0	little	little
0.005	almost 0	moderate	moderate
0.0035	trace	marked	marked
0.0025	little	almost complete	almost complete
0.0015	moderate	complete	complete
0.001	marked	"	"
0.00075	"	"	"
0.0005	"	"	"
0.00035	"	"	"
0.00025	almost complete	"	"
0.00015	complete	"	"

<sup>1</sup>The solution of cholesterin was made by diluting 1 cc. of a saturated solution of cholesterin in hot methyl alcohol, with 9 cc. 85% salt solution

A third difference between cobra amboceptor and the finished lecithid is seen in the behavior toward high temperature. The aqueous solution both of the primary and the secondary cobra lecithid is far more stable than solutions of the amboceptor alone. The former can be heated to 100° C. for six hours without any particular loss in power, while the amboceptor of cobra venom loses its action if heated to 100° C. for only thirty minutes. Obviously this is to be explained

by assuming that the combination has become firmer by the entrance into it of the lecithin molecule.

There is a fourth point of difference, the behavior toward the snake-venom serum discovered by Calmette. The finished lecithid is affected far less by this serum than is the cobra amboceptor. We shall discuss this in a later article.

In contrast to these differences the behavior of cobra lecithid and cobra amboceptor + lecithin toward cholesterin is similar. We have already mentioned that cholesterin possesses the power to inhibit the hæmolysis by means of cobra venom. The same is true in hæmolysis by means of the finished lecithid, although quantitatively to a less degree. (See Table III opposite.)

#### IV. The Lecithids of Several Other Poisons.

Naturally it was of considerable interest to see whether this peculiar formation of lecithid (thus far without parallel in chemistry) was confined to cobra venom, or extended also to other poisons. The following poisons, which we owe to the courtesy of Dr. Lamb, Prof. Calmette, Dr. Kinyoun, Dr. Dowson, and Prof. Kitasato, have therefore been studied by us for this purpose:

1. *Bothrops lanceolatus*;
2. *Daboia Russellii*;
3. *Naja haye*;
4. *Kerait*;
5. *Bungarus fasciatus*;
6. *Trimeresurus anamalisensis* (Hill viper);
7. *Trimeresurus Riukiuanus* (Japan);
8. *Crotalus adamantus*.

In a subsequent article we shall discuss the behavior of these poisons toward different species of blood-cells. For the present, however, we may say that all of these poisons, on the addition of sufficient lecithin, dissolve the blood-cells examined by us, namely, those of man, guinea-pig, rabbit, ox. With the exception of two poisons (*Bothrops lanceolatus* and *Trimeresurus anamalisensis*) the absolute quantity of poison necessary to effect solution, an excess of lecithin being present, is about the same for all species of blood examined; 0.003 grm. are sufficient to dissolve 1 cc. of a 5% suspension. The

*Bothrops* poison is ten times weaker, and that of *Trimacrusurus anamalensis* twenty-five times. This observation made the formation of a lecithid seem probable. As a matter of fact it was easy, by means of the method above described, to prepare a solid lecithid which contained the entire hamolytic power of the poisons.<sup>1</sup> Hence we believe that in general all hamolytic snake venoms are of the amboceptor type and possess a lecithinophile group, the occupation of which by lecithin gives rise to the hamolytic action. In fact it seems as though in the last analysis the factor which determines the type of the hamolytic action of snake venom was principally this lecithinophile group.

A fact which goes to support this view is the observation that several of the poisons examined by us probably differ in their haptophore group, which unites with the receptor of the blood-cells. Thus Lamb<sup>2</sup> has shown that the Daboia amboceptor, unlike the cobra amboceptor, is not inhibited in its action by Calmette's serum. The same is true for *Bothrops*, *Crotalus*, and *Trimacrusurus Reinkenianus*, whereas the poisons of *Bungarus* and *Naja haje* are similar to the cobra venom so far as their behavior toward the serum is concerned.

It is quite possible, therefore, that the differences in the various types of poison are only differences in the haptophore group, while the characteristic lecithinophile group is identical in all cases.

It was important to see whether in animals other than snakes poisons are present which are capable of forming lecithids. We therefore next studied the poison of the scorpion, choosing this because Calmette<sup>3</sup> had already shown that the acute fatal action of scorpion poison could be inhibited by the snake-venom serum, a fact indicating a certain analogy between the toxic components of scorpion poison and snake venom.<sup>4</sup> We were able to determine that the scorpion poison by itself exerts only a slight hamolytic action on guinea-pig blood-cells, leaving other species of blood-cells unaffected. On the addition of lecithin, however, it exerts con-

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<sup>1</sup> In conformity with its weaker action *Bothrops* poison yields only a tenth the lecithid obtained from the other poisons, and the poison of *Trimacrusurus anamalensis* only one twenty-fifth.

<sup>2</sup> Lamb, Scientific Memoirs, Medical and Sanitary Dept., Govt. of India, 1903, No. 3.

<sup>3</sup> Calmette, Ann. de l'Institut. Pasteur, 1895, No. 4.

<sup>4</sup> For this scorpion poison we are much indebted to Prof. Treub, Director of the Botanical Garden in Buitenzorg.



siderable solvent action on all the different species of blood examined by us. Its action is about one twentieth as strong as that of cobra venom. (See Table IV.)

TABLE IV.

ACTION OF SCORPION POISON WITH AND WITHOUT THE ADDITION OF LECITHIN.

Amounts of the 0.2% Solution of Scorpion Poison cc	1 cc. 5% Ox Blood.	
	+ 0.2 cc. 0.1% lecithin.	Control without lecithin.
1 0	complete	0
0 75	"	0
0 5	"	0
0 35	"	0
0 25	"	0
0 15	"	0
0 1	"	0
0 075	"	0
0 05	"	0
0 035	"	0
0 025	"	0
0 015	almost complete	0
0 01	moderate	0
0 0075	little	0
0 005	trace	0
0 0035	"	0
0 0025	faint trace	0
0 0015	0	0

Corresponding to this behavior we succeeded in actually producing a typical lecithid from scorpion poison by following the usual procedure.<sup>1</sup>

All this leads us to the view that the essential character of the hæmolytic cobra venom is due not to the haptophore group, but finally to the lecithin anchored by the blood-cells by means of a lecithinophile amboceptor. Now we know that lecithin is present in every red blood-cell, and this seems apparently to contradict the fact determined by us experimentally that the lecithin is the cause of hæmolysis. This contradiction, however, is merely apparent, for we need only assume that by the aid of the cobra venom

<sup>1</sup> It is probable that the poison of a fish, *Trachinus draco* (see Briot, Journ. de Physiol. et de Pathol. gén. 1903, No. 2), is also capable of forming a lecithid; at least a statement of Briot speaks in favor of this, namely, that the hæmolytic agent in the *Trachinus* poison can be activated by a serum which has been heated to more than 60° C.

the lecithin is brought into proximity with cell constituents other than those normally in its proximity. In other words, we are dealing with the deleterious action of a vitally important substance which has been forced into the *wrong place*. This conclusion is made plain if we bear in mind the fact that in the blood-cells primarily susceptible to cobra amboceptor, the hemolytic action depends not on the addition of new lecithin, but on a transposition of the lecithin preformed in the cell, due to the anchoring of the cobra amboceptor.

## XXXVII. THE CONSTITUENTS OF DIPHTHERIA TOXIN.<sup>1</sup>

By PAUL EHRLICH.

THE Festschrift, published at the opening of the Serum Institute in Copenhagen, contains a study by Arrhenius and Madsen<sup>2</sup> which deals mainly with the neutralization phenomena of toxin and antitoxin. We must all rejoice that Madsen has succeeded in interesting so excellent a physical chemist in this question, especially as I had tried unsuccessfully for years to secure the interest of physical chemists in Germany. In the present state of scientific knowledge we shall for the present have to give up our attempts to isolate the toxins in pure form. For the same reason also in the analysis of the relations between toxin and antitoxin we cannot conform to the ordinary methods of the chemist working with the balance. On the other hand, the study of toxin and antitoxin is of too great practical importance for us to wait idly for years or decades until chemistry is so far advanced. We must, therefore, content ourselves with the slight means at our disposal, applying these, however, in all directions in order to gain as great an insight into this complicated subject as the present state of our knowledge permits. I had applied myself to this problem for years and come to the conclusion that the only way to approach it was by an exact quantitative study of the neutralization phenomena. Particularly in *partial neutralization* I believed I had found a method by which we could gain an insight into the most intricate constitution of the toxins. To my regret high authorities pronounced this method as incorrect and of no avail. I am all the more pleased, therefore, to see that so high

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<sup>1</sup> Reprint from the Berl. klin. Wochenschr. 1903, Nos. 35-37.

<sup>2</sup> S. Arrhenius and Th. Madsen, Physical Chemistry applied to Toxins and Antitoxins, Festschrift ved indvielsen af Statens Serum Institute, Copenhagen, 1902. (This is to be had in English text, Copenhagen, 1902.)

an authority as Arrhenius recognizes my method as correct in principle and proceeds along the same lines.

The study of Arrhenius and Madsen deals principally with tetanolysin, the hæmolytic poison discovered by me in tetanus toxin. Tetanolysin and tetanospasmin differ from each other in their haptophore groups, as a result of which each possesses a particular antibody in the tetanus serum of the market. Madsen studied this tetanolysin in my Institute, and found that when it is gradually neutralized with increasing amounts of antitoxin, the same definite amounts of antitoxin first added neutralize far more poison than subsequent additions. Because of this and also because of other reasons (attenuation, phenomena during neutralization) Madsen concluded that several poisons of different affinities were present.

On taking up these studies in tetanolysin Arrhenius and Madsen obtained practically the same results, and these authors succeeded in constructing a formula for the action of antitetanolysin on tetanolysin which conforms to the law of Guldberg-Waage. Based on this they next attempted to determine similar relations in the case of very simple blood poisons. This had already been done by Danysz, but the method was open to criticism. Arrhenius and Madsen chose a weak base and an acid (ammonia and boric acid) as hæmolysin and antihæmolysin. It was found that in these the neutralization phenomenon is very similar to that of tetanolysin and antilyysin, from which they concluded that in the neutralization of toxins and antitoxins we are dealing with reactions between simple substances of weak affinities.

In this connection they express themselves as follows: "The last-mentioned curve gives a fairly accurate picture of the neutralization of ammonia with boric acid. In the investigation of ammonia as hæmolysin a spectrum analogous to that of toxin or tetanolysin (Fig. 3) could have been constructed; the following conclusion could also perhaps have been drawn: One part of boric acid (antitoxin) added to ammonia neutralizes 50% of this base; if two parts are added it neutralizes 66.7%; if three parts, 75%; and four parts, 80%. From this it follows that since the respective amounts 50, 16.7, 8.3, and 5% are each time neutralized by the same amount of boric acid, the amount first neutralized is three times as toxic as the amount next neutralized, this again twice as toxic as the next after it, which in its turn is one and one-half times as toxic as the following, etc. In other words, ammonia is not a simple substance,

but consists of several constituents of different toxicity (and these toxicities bear a simple reciprocal relation to each other). Of these constituents the toxin possessing the highest chemical affinity is neutralized first.

A similar conclusion has actually been drawn in the case of toxin; the toxin first neutralized (the strongest) has been called prototoxin, the next deuterotoxin, the next tritotoxin, etc. The final very weak toxins are called toxones.<sup>1</sup>

The findings of Arrhenius and Madsen are thus seen to be directly opposed to my statement that diphtheria poison is composed of several constituents. In view of the exceeding importance of the subject I cannot avoid entering the discussion and state the reasons which cause me to maintain my views absolutely and without any modification.<sup>1</sup>

The new views of the authors in question will doubtless lead many to wonder how I could err in so simple a matter and employ complicated theories when the simplest conceptions of chemistry would have sufficed. It must seem strange that I, who have followed this subject for years and have busied myself especially with chemical studies, should have failed to discover this very ready explanation. As a matter of fact, however, I too began with the conception, now held by Arrhenius and Madsen, that in the union of toxin and antitoxin we were dealing with a phenomenon of incomplete neutralization. A more thorough analysis of *diphtheria poison* (my publications refer only to this poison) compelled me, however, to adopt more complex explanations.

At the very outset of my investigations I discovered that tetanolyisin and its antitoxin possess weak affinities, and I devised the tech-

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<sup>1</sup> Gruber, whose experiments especially devised to refute my theory I was able to show were incorrect, has employed the opportunity to side with Arrhenius and Madsen, and to announce that their observations "will give this entire spook of side-chain theory its quietus." No one who knows anything about this subject needs be told that the question as to whether diphtheria poison is made up of one or more substances has nothing to do with the side-chain theory. When I formulated this theory I too believed the diphtheria poison to be a simple substance, and when subsequently I felt compelled to differentiate several components in the poison I always emphasized that the separate components differed only in their toxophore group and were similar so far as the haptophore groups were concerned, the groups which give rise to antitoxin formation (see my reply to Gruber in Münch. med. Wochenschr 1903, Nos. 33 and 34).

nique of my experiments accordingly. At that time I stated in connection with this tetanolsin that the union of toxin and antitoxin proceeds more slowly in dilute solutions than in concentrated, and that the process is hastened by heat. How feeble the combining affinities of tetanus toxin and antitoxin are can be seen from the following experiment devised over eight years ago: If a given, not very concentrated, mixture of serum and toxin is allowed to stand for two hours it will be found that the action of the serum is forty times as great as when the mixture is employed immediately. Whether the optimum of neutralization is thus reached is difficult to say. The determination of the exact limits fails because of the fact that the poison rapidly decomposes in watery solutions, especially if these be dilute. One constantly faces either of two difficulties: insufficient union on the one hand and decomposition of the poison on the other.<sup>1</sup>

With diphtheria poison, on the other hand, the affinity of the toxin for the antitoxin is much greater. As is well known, these substances unite so rapidly that even the time for combination prescribed in the test—fifteen minutes—is still unnecessarily long. Hence, even if I admit that the union of tetanolsin and antilysin is comparable to the neutralization of a weak base by a weak acid, I shall in the following pages show that the affinity of diphtheria toxin and antitoxin is very great, comparable perhaps to that between a strong acid and base. In accordance with this also I am convinced that the neutralization of diphtheria toxin by antitoxin proceeds in the form of a straight line and not in that of a curve. This, then, constitutes my first objection to the general deductions drawn by Arrhenius and Madsen from their particular findings. Just as it is impossible to apply the results of the neutralization of boric acid and ammonia to every combination of acid and base, so it is impossible to apply the experiences with tetanolsin to the doctrine of toxins in general.<sup>2</sup>

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<sup>1</sup> When, then, years ago, in spite of these unfavorable conditions, I proposed the study of tetanolsin to Thorvald Madsen, this was but a makeshift necessitated by the lack, at that time, of suitable hæmolysins. At present a number of such substances are available, such as arachnolsin and snake venom. These are very stable and far better suited for exact determination since the factor of decomposition is absent.

<sup>2</sup> I should like to mention that recently Dr. Kyes has discovered that in snake venom also the neutralization with antitoxin proceeds with high affinities and in a straight line.

If, therefore, the affinity between diphtheria toxin and antitoxin is so great, we shall have to ascribe the irregular course of the neutralization process to other factors than those assumed by Arrhenius and Madsen.

### Diphtheria Toxins.

In order to understand what follows it will be necessary to speak of some of the main principles of toxin-antitoxin analysis. As is well known diphtheria toxin is the bouillon fluid in which the diphtheria bacilli have grown, and to which they have given up their toxic secretory products. In order to determine the toxicity we make use of guinea-pigs. The lethal dose (L. D.) is that amount of poison which will surely kill a guinea-pig weighing 250 grammes on the fourth day. In order to determine the relations between toxin and antitoxin it is best to start from the serum because this can be preserved constant by means of the methods devised by me (vacuum, drying). This dry serum also serves as the standard for the official titration. The immune unit (I. E. = Immunitäts Einheit) is, of course, an arbitrary quantity which originated by terming that amount of antitoxin a unit which just neutralized 100 L. D. of a poison that happened to be available at the time, so that the mixture when injected did not produce even the slightest trace of illness (either general or local reaction).

If one mixes one immune unit of serum with graduated amounts of poison, two limits may be obtained. One of these is termed limit zero ( $L_0$ ), and corresponds to the quantity of poison which is completely neutralized by 1 I. E. The other is limit death ( $L_+$ ) and corresponds to that quantity of poison which on the addition of 1 I. E. is so far neutralized that only just one L. D. remains. Of these two limits the  $L_+$  is very easily and accurately determined so that it serves as a measure in testing the potency of the diphtheria serum. This limit signifies nothing more than that of  $x$  L. D. present, 1 I. E. neutralized  $x-1$  L. D., so that just 1 L. D. remains free and leads to the death of the guinea-pig in four days.

A priori one might have expected that the number of lethal doses which are neutralized by 1 I. E. is always the same in poisons from different sources. The only difference which one would have expected would be that in different poison solutions, the volume in

which a given number of L. D. were contained would vary from case to case, depending on the varying quantity of poison produced by the bacilli.

Closer investigations, however, showed that in reality the conditions are entirely different, the number of L. D. contained in L<sub>t</sub> varying enormously in different toxic bouillons. In poisons which have been analyzed the figures have fluctuated between 15 and 160. Since it had been shown, especially by myself, that the neutralization of toxin-antitoxin rests on a chemical basis, this result could only be explained by assuming that the diphtheria bouillon, in addition to the toxins, contained other non-toxic substances which were able to combine with antitoxin just like the diphtheria toxin. I deemed it to be of the highest importance to clear up this mystery experimentally, and therefore subjected a number of different poisons (some freshly derived, others precipitated with ammonium sulphate, and still others which had been kept for a long time) to comparative analyses. In the course of these it was found that the non-toxic substances, which still possess combining properties, increase as the toxic bouillon ages, and I therefore studied these changes in the poisons genetically at various stages.

I emphasize this part of my method because the casual remark by Arrhenius and Madsen<sup>1</sup> that my results were derived mainly from a study of decomposed poisons might readily be misconstrued and give one the impression that in my investigations I had not been especially careful. I may at once add, however, that my most valuable results were obtained by studying the course of this decomposition, but this, of course, corresponds entirely with the methods of chemistry. It is impossible to gain an insight into the constitution of highly complex combinations by means of an analysis which leads only to the compact formula. This can only be gained by the careful decomposition of the substance to be studied. Whatever knowledge we possess regarding the constitution of sugars, uric acid derivatives, alkaloids, etc., is due mainly to the decompositions intelligently carried out, and a careful study of their products. Of course, the decomposition must not give rise to secondary reactions which could obscure the results; this might be the case if strong acids or a high temperature were employed. The decomposition must be quantitative and of moderate intensity. The following observations will show that this is especially the case in the spontaneous

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<sup>1</sup>l. c.



attenuation of the toxins, which occurs at room temperature and without any further chemical manipulation.<sup>1</sup>

*It has been found that the bouillon on standing can preserve its neutralizing property intact, and often actually does so, while the toxicity is considerably decreased.* Observations of this kind have been made by myself and Madsen for diphtheria poison, by Jacoby for ricin, by Myers for snake venom, and recently by Arrhenius and Madsen for tetanus poison. This phenomenon, which in many cases is quantitative, is most readily explained by assuming that the poison molecule contains two functioning groups. One, the "haptophore group," combines with the antitoxin and in the animal body effects the combination with the tissues; this group is quite stable. The other, the "toxophore group," effects the true poisonous action; it is comparatively readily destroyed. In my opinion the transformation of toxin into toxoids by the destruction of the toxophore group is the key to a correct understanding of my conception of antitoxic immunity and the subject of toxins.<sup>2</sup>

If we see, for example, that in spite of decreased toxicity the constants of neutralization  $I_+$  and  $I_0$  remain entirely unchanged, it follows, in my opinion, that two important deductions can be made. The first is one which I have always drawn, namely, that in normal toxoid formation not brought about by chemical additions, the number of haptophore groups suffers no loss. This behavior, however, also seems to indicate that in toxoid formation the affinity of the haptophore groups for the antitoxin is in no way changed. I may be permitted to elucidate this by means of a chemical example. Tetramethylammoniumhydroxid is a very strong base (like KOH) which through suitable procedures (heating, etc.) is transformed into the

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<sup>1</sup> Obviously these poisons can also be attenuated through chemic or thermic influences, but the decomposition in that case takes place rapidly and with destruction. In my investigations, therefore, I have never made use of these methods, but have kept to the moderate changes which occur spontaneously in the toxic bouillon on standing.

<sup>2</sup> At the outset of the modern study of immunity, von Behring, Aronson, and others had observed that an active immunity could be brought about particularly through attenuated, modified poisons. At that time, however, it was very difficult to appreciate these relations, and so in the year 1894 we find a high authority, as a result of his investigations, denying the existence of modified poisons, although he had previously assumed their existence. The results, which had been obtained with immunization, he ascribed, not to the presence of modified poisons, but exclusively to a dilution of the poison.

far less basic trimethylamin, methyl alcohol being split off in the process. Let us take a certain definite quantity of tetramethylammonium hydroxid, say 20 molecules, and determine the quantity of boric acid which will just suffice for complete neutralization, as shown by a suitable indicator. On changing the ammonium base into the tertiary amin (a change which we shall assume to be complete) we shall find that a larger quantity of boric acid is necessary for neutralizing the tertiary amin. In other words, there has been a change in the position of the neutral point, although the number of basic radicals remains the same. This necessarily follows from the decrease in affinity brought about by the transformation.

The reverse will take place if a weak base is transformed into a stronger one. A change in the position of the neutral point will occur even if the transformation is only a partial one, i.e., does not affect the entire number of molecules. If, however, in spite of an extensive formation of toxoid, we find the test limits unchanged, we can only conclude that any considerable change in affinity has not occurred. We shall subsequently learn of another fact, which affords conclusive evidence of the correctness of these views.

Our next problem will be to study the influence of the toxoids on the neutralizing process. To begin, it should be remarked that the bacterial poisons with which we are dealing are not, as a rule, pure poisons. By this, of course, I do not mean to deny that pure poisons can occur. If the toxophore group possesses considerable resistance so that it is not affected by the processes used in its production (keeping in the incubator for weeks, etc.), it will be possible to obtain poisons which contain only toxins and no toxoids. Such a result, however, can probably only be counted on in a small number of isolated cases, and is not obtained as a rule. So far as diphtheria poison is concerned, of which I have made a special study, I have never yet, among a large number of specimens examined, found a single one free from toxoids. In estimating the degree of purity one proceeds by finding in various poisons how many fatal doses (L. D.) are neutralized by one immune unit (I. E.). The maximum value in the poisons at my disposal was 130, but Madsen has described a poison in which the  $L_+$  dose contained 160 L. D. But even this poison, as I shall show later,<sup>1</sup> merely *approached* the character of a pure poison.

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<sup>1</sup> It is especially important that even diphtheria poisons which have been

Naturally the poisons whose toxophore groups are very labile will be the least pure. This is especially true in tetanus poison, which is far more readily destroyed than diphtheria poison. In the former, several hours' standing of an aqueous solution suffices to give rise to toxoid formation. It is all the more probable, therefore, that the toxin produced in the usual manner by keeping the culture in the incubator for eight days contains a considerable admixture of toxoids. In the precipitation with ammonium sulphate these toxoids, of course, are present in the resulting solid product.

A dry poison of this kind, such as I placed at Madsen's disposal for his experiments, can, of course, keep for a long time unchanged provided it is carefully preserved; the primary content of toxoid, however, also remains unchanged.

For this reason I believe that the assumption of Arrhenius and Madsen, that the tetanus poison used by them was a pure poison, since it did not change, is entirely unwarranted. It is even possible that this particular specimen contained far more toxoids than the old toxin solutions which I had employed.

In pure chemistry in carrying out exact mathematical determinations it is a general principle that the substance be either absolutely pure or at least that its degree of purity be exactly determined by analysis. In determining the molecular weight of an element, a great deal of preliminary work (recrystallization, etc.) is required in order to obtain the original material as pure as possible. If this cannot be done, as, for example, in the case of hydrogen peroxide, or ozone, a quantitative study requires at least that the exact percentage of pure substance contained in the mixture be known. It is hardly necessary to say that these principles should, as far as possible, be applied to the study of toxins. In these substances also one should know the degree of purity before attempting any exact investigations. But just in this domain, where it is impossible to isolate the substances, this task is uncommonly difficult. It required a year's most tiresome and monotonous labor before I was able, by means of very exact determinations of all kinds of poisons, to approach this problem. At that

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produced in a very short time (three to four days in the incubator) are not free from toxoids. In one such poison (No. 9 of the titration series) I found 123 L. D. in  $L_4$ . I was therefore greatly pleased recently to hear from Dr. Louis Martin, who has had such wide experiences in this direction at the Pasteur Institute, that in his fresh poisons he never saw the figure 200 L. D. in  $L_4$  reached.

time I gained the impression that a pure poison must be so constituted that one I. E. fully neutralizes exactly 200 L. D.<sup>1</sup> Later on I shall show that by means of the "spectrum" analysis I have succeeded in verifying this figure.<sup>2</sup>

The discovery of this number, 200, led me to represent the constitution of diphtheria poison by means of a "spectrum" which is divided into 200 segments, each of which corresponds to a toxin, toxoid, or toxon equivalent. This scheme is not, as some have assumed, a mere makeshift, but is the expression of knowledge laboriously attained. This graphic reproduction shows at a glance how much toxin or toxoid is neutralized by each combining unit of antitoxin. Such a reproduction possesses so many advantages over the curve used by Arrhenius and Madsen that I shall not hesitate a moment in retaining the spectrum method for diphtheria poison. By its means one obtains a view of the entire process of neutralization.<sup>3</sup>

It may be well at this point, by means of a suitable chemical illustration, to elucidate the influence which such admixtures of toxoid exert in the titration of alkaloids. In doing this it will be best to proceed on the following assumptions. An alkaloid acts hæmolytically when in the form of free base, but not when in the form of a salt.<sup>4</sup> The base would then correspond to the toxin. The analogue of the toxoid would then be an alkaloid which exerts no deleterious action either as such or in the form of a salt. The antitoxin would be represented by any acid, e.g., hydrochloric acid. Under these conditions the mixture of the two alkaloids can be titrated biologically (by determining the hæmolytic power at any point) by means of an acid exactly as a toxin solution containing toxoid by means of its antitoxin.

Let us assume that the toxic alkaloid *A* as well as the atoxic *B* possesses so strong an affinity for hydrochloric acid that neutralization is effected to within a very small fraction. A solution of  $\alpha$  molecules *A* would then correspond to the pure toxin, while mixtures of

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<sup>1</sup> It is self-evident that each toxin-combining unit can be replaced by an equivalent amount of less toxic or non-toxic substances possessing combining properties (toxones, toxoids).

<sup>2</sup> The poison studied by Madsen, therefore, which contained 160 L. D. in  $L_t$ , corresponded to a purity of four-fifths.

<sup>3</sup> See also page 552.

<sup>4</sup> This is probably the case with solanin, whose hæmolytic power is inhibited by the addition of acid salts (Pohl) or of free acids (Hédon, Bashford).

*A* and *B*:  $\frac{\alpha}{2} + \frac{\beta}{2}$  or  $\frac{\alpha}{4} + \frac{3\beta}{4}$  represent analogues of solutions containing also toxoids. In all of these mixtures the end point of neutralization will be practically constant. If, however, the affinities of *A* and *B* for hydrochloric acid are not exactly equal the neutralization will proceed in a straight line only if we are dealing with the pure alkaloid. In all other cases it will follow the course of a curve whose character, of course, is dependent on the relative amounts of the two components.

This problem of the simultaneous neutralization of two alkaloids has been studied in suitable cases by J. H. Jellet. Let us take the neutralization of quinine and codein with hydrochloric acid, in which the coefficient of equilibrium  $K=2.03$ . For the sake of simplicity I have assumed this to be 2.0. In order, furthermore, to have the conditions as simple as possible, let us take as an example a mixture of 100 molecules quinine and 100 molecules codein. These will then be neutralized by 200 molecules hydrochloric acid. By means of the formula devised by Jellet one next determines how much quinine is transformed into the salt by each successive addition of one-tenth the entire neutralizing dose (20 molecules HCl). It will be found that the first tenth neutralizes 13 and the last tenth 7 molecules of quinine, while the course of the neutralization of the quinine is itself entirely uniform. If another combination is taken, in which the second alkaloid possesses a weaker affinity, so that  $K=10$ , it can easily be calculated that under these circumstances the first tenth hydrochloric acid neutralizes 17.8, the last tenth only 3 molecules of quinine. On representing these reactions graphically we shall obtain curves entirely similar to those representing the neutralization of a weak base with a weak acid, and it would probably not be difficult to find a combination of alkali and acid whose curve corresponds to the alkaloid curve mentioned.

Hence, if such a mixture of alkaloids together with the appropriate neutralizing agent (acid) were given one for a biological titration, and if, furthermore (to make the analogy with toxin-antitoxin determination complete), the employment of any additional chemical aids was barred, the neutralization curve obtained under such stringent conditions could easily give the impression that one were dealing only with the neutralization of two substances possessing weak affinities. Nevertheless, even under these limitations, it is possible to learn the true conditions if, as I have done, one does not confine one's

self to a single mixture, but analyzes a great many different mixtures in which the relation of toxin-alkaloid and toxoid-alkaloid varies.<sup>1</sup>

It is all the more surprising that in the analysis of the constitution of poisons Arrhenius and Madsen have not studied the question from this point of view because they do not at all neglect the existence of toxoids. Apparently this is because of a slight misunderstanding, for these authors proceed exclusively on the assumption that in toxoids one is dealing with prototoxoids, i.e., with toxoids which possess a higher affinity for the antitoxin than does the toxin. In fact, one can easily observe that the formation of prototoxoids affects the end point of the titration but little. This I had predicted in my first study on the evaluation of diphtheria serum. Let us assume, for example, that a mixture of 1 equivalent hydrochloric acid (prototoxoid) and 3 equivalents prussic acid (toxin) is neutralized by a strong base. In that case the hydrochloric acid will be neutralized first, after which the neutralization of the prussic acid will proceed very much the same as though only prussic acid were present.

We must now see whether diphtheria poisons, such as I have investigated, contain other toxoids besides prototoxoids. The material at hand makes the decision of this point very simple. In four poisons containing a prototoxoid zone (of which two were published by myself and two by Madsen) I have calculated the relation of prototoxoid and toxoid to toxin. In doing this I have regarded exclusively the  $L_+$  dose, and so eliminated the toxons which would otherwise still more increase the toxoid figure.

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<sup>1</sup> In the very simple example of two alkaloids just mentioned two determinations of different mixtures would permit the calculation. In my opinion no definite conclusions as to the constants of the toxin can be drawn from the analysis of one particular toxin containing toxoid. Arrhenius and Madsen analyzed two different tetanus poisons, one of which had undergone toxoid modifications through years of preservation as a dry substance, while the other had suffered similar modifications through several days' standing of the solution. The authors calculated from their experiments that in the one case the constant of dissociation had been increased 50%, in the other ten times. In view of what has just been stated this calculation, which leaves out of account the presence of toxoids, cannot be regarded as conclusive. The divergence of the constants could easily be due exclusively to the presence of toxoids, and these, in view of the different methods by which the poisons were attenuated, could be different in the two cases. I may also add that in the toxoid formation of diphtheria toxins I am convinced that the toxin groups which remain do not suffer any change in their affinity.

Poison.	For 100 Parts of Toxin there are	
	Prototoxoid, Parts.	Toxoid, Parts.
A Madsen	160	400
C Madsen	79	59
IV Ehrlich	82	200
V Ehrlich (4th phase)	77	131

This table shows that the four poisons contain considerable amounts of toxoids in addition to the prototoxoids. The affinity of these toxoids is more or less small, as can be seen from the curves plotted by Madsen and myself. From this it follows that in the interpretation of the results obtained by neutralizing diphtheria poison due attention must be paid to the decisive influence exerted on the course of the partial neutralization by the toxoids notoriously present in such considerable amounts. It is incorrect, therefore, to refer the decreased binding of antitoxin, such as is seen in the tritotoxoid zone, to the boric acid-ammonia scheme.

It will be well, by means of a concrete example, to study somewhat more in detail the course of this toxoid formation. For this purpose I shall select a poison which I have already described in my publication on the constitution of diphtheria poison<sup>1</sup> as Poison No. 5. At that time I briefly gave the spectrum and the constants based on the investigations which I and my friend Dönitz had carried out. In this poison the conditions were most interesting and yet extremely simple: The  $L_0$  dose was 0.125 cc.; the  $L_+$  dose 0.25 cc., that is, just twice as much. The L. D. was 0.0025 cc., so that the  $L_0$  dose contained exactly 50 L. D. and the  $L_+$  dose exactly 100 L. D. These facts caused us to make the thorough analysis. This poison, as is so often the case, suffered certain transformations, whereby it became weaker. These changes occurred in three phases characterized by the formation of different kinds of toxoids. The spectra of these phases are as follows (Fig. 1).

The phases in which the content of toxin shows itself are I, II, and IV; phase III, which deals with the toxons, will be considered in a separate chapter.

As a result of all my experiences with similar poisons, as well as

from a direct determination, it follows that the first phase must have represented a pure hemitoxin which reached exactly to 100 (see illustration). Accordingly each  $\frac{1}{200}$  I. E. (=1 combining unit) successively added to the L dose takes away  $\frac{1}{2}$  L. D. from the fatal doses

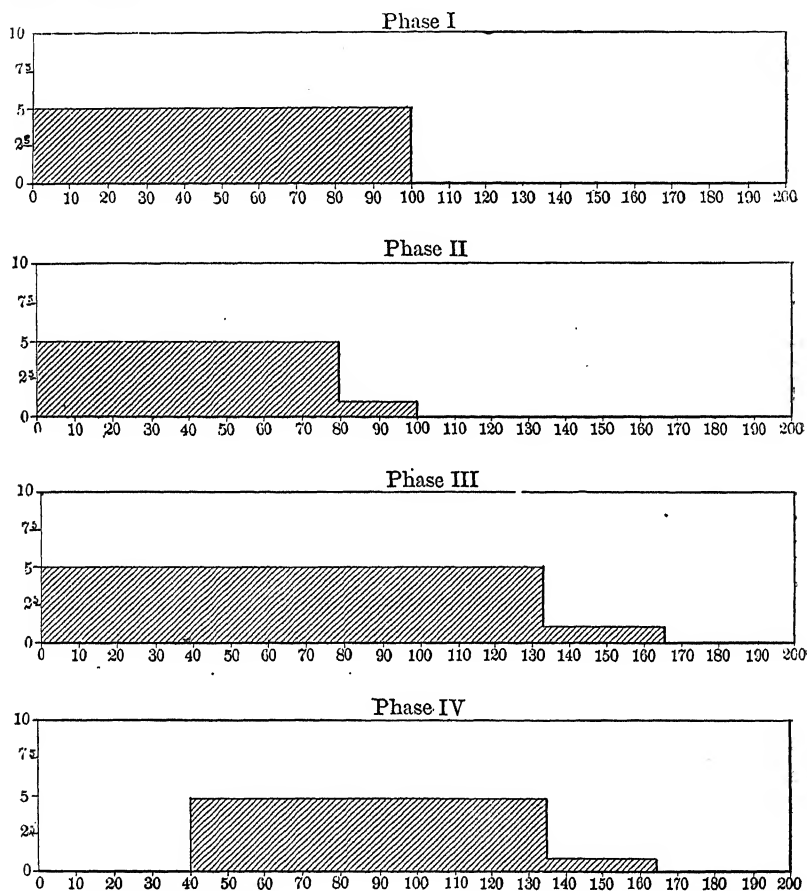


FIG. 1.

contained in  $L_0$ , and this all occurs within the first hundred antitoxin doses added. Amounts of antitoxin beyond this have no further influence on the toxin (death, necrosis), but affect only the toxin.

A fact to which I attach particular significance is that the hemi-

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mean



toxin reaches just up to the 100 limit and shows no trace of any gradual decline. This follows from the determination of the  $L_+$  dose, as can be seen from the following analysis.

Given a poison in which, in the  $L_0$  dose, the hemitoxin zone reaches exactly to 100, how large will the  $L_+$  dose be?  $L_+$ , i.e., the amount of poison which on the addition of 200 combining units still leaves 1 L. D. free, will be reached when 200 equivalents of hemitoxin are present. We shall therefore have to multiply the  $L_0$  dose of the poison by  $\frac{202}{100}$  in order to obtain the  $L_+$  dose. If we carry out this multiplication we obtain an  $L_+$  dose of 0.253, which agrees very well with the value actually found, 0.25 cc.

Thus the important fact is demonstrated that in this case the neutralization of the diphtheria poison by antitoxin proceeded exactly the same as the neutralization of a strong acid by a strong base. Here then the course of the reaction is represented by a straight line and not by a curve.

Further evidence for the view that in this poison the hemitoxin extended right up to the limit 100 is furnished by phase II. Here we see a simultaneous increase of the  $L_+$  dose and a decrease of the toxicity manifesting themselves by the fact that the L. D. increases from 0.0025 to 0.003 cc., so that the number of L. D. contained in the  $L_0$  dose has decreased from 50 to 42.

This increase of the  $L_+$  dose amounted to about 0.26 cc. and from it, by means of the simple calculation already mentioned, it can be shown that toxoid formation took place in the end zone of the toxin, the "tritotoxoid zone," as I term it.

Let us assume that the end zone (which before as well as after the second phase extended to 100) contains a toxoid mixture of  $\frac{1}{10}$  toxicity instead of the hemitoxin. In order to reach the  $L_+$  dose in this case we must multiply the  $L_0$  dose by  $\frac{210}{200}$  and not by  $\frac{202}{200}$ , as was the case with hemitoxin. On carrying out this calculation,  $L_0$  being 0.125, we get  $\frac{0.125 \times 210}{100} = 0.2625 = L_+$ .

In the determination made at that time I actually found the  $L_+$  dose to be 0.26, but noted "a little over." That the tritotoxoid zone possessed a toxicity of  $\frac{1}{10}$  was shown by the subsequent analysis by means of partial neutralization, for near the end, a zone of 18-20

tritotoxoid of exactly  $\frac{1}{10}$  toxicity was found. It should be emphasized that the fatal doses which disappeared in the deterioration were found in the form of toxoids in the tritotoxoid zone.

These investigations show that these changes are due exclusively to the fact that a part of the toxin has become transformed into toxoids; in fact into toxoids which are neutralized after the main portion of the toxin, and which, therefore, must possess less affinity. If we were to represent this phase by means of a curve according to the method of Arrhenius and Madsen, we should observe a marked flattening of the curve in the tritotoxoid zone. This, however, is not the expression of the weak affinity of the diphtheria toxin, or of the neutralization dependent thereon. It is to be ascribed with absolute certainty solely to the presence of toxoids and their appearance in place of toxin molecules which have disappeared.

I shall discuss phase III later, merely remarking at this time that in this phase, 80 out of 100 parts toxon have disappeared. The  $L_0$  dose of 0.125 cc. now contains only 120 combining units instead of the 200 units (toxin and toxon) originally present. Corresponding to this, therefore, the  $L_0$  dose, which must contain 200 combining units, increases from 0.125 cc. to 0.21 cc. In this third phase the toxin zone has not suffered any essential change. The  $L_+$  dose has accordingly remained constant at 0.26 cc. Because of the new  $L_0$  dose made necessary by the loss of toxon, the spectrum representing this phase shows a much wider toxin zone than the previous one. The toxin-toxon boundary has been moved from 100 to 166.

In phase IV,  $L_+$  remained 0.26 cc., but the toxicity decreased, the L. D. increasing gradually from 0.003 cc. to 0.004 cc. During the course of these changes 22 L. D. had disappeared from the  $L_0$  dose of phase III.

The fate of these 22 L. D. is made plain by the spectrum which I constructed at that time. In this I found an extended prototoxoid zone which included the first 40 combining units of the spectrum, sufficient, as can be seen, to explain the loss of toxin which had occurred. I desire to call particular attention to the fact that no loss of combining groups had occurred despite the slight increase of the  $L_+$  dose.<sup>1</sup>

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<sup>1</sup> A superficial glance might lead one to suppose that the fact that the  $L_+$  dose of 0.25 cc. in the first phase had become increased to a little over 0.26 cc.,

This behavior shows that on standing there is not, for example, a marked destruction of the poison, but merely a slight chemical change affecting only the toxophore and not the haptophore group. It would be improper, therefore, to speak of the poison "spoiling."

The observations on the origin in the various forms of toxoid are particularly important.

In the first phase of toxin formation, there was a development of toxoids of weaker affinity for the antitoxin, while during the second stage, toxoids of greater affinity developed. Occupying a position between these two opposing poison modifications is the hemitoxin fraction, and this has remained intact. We are thus really forced to arrange these three poison constituents, according to their affinity, as prototoxoid, deuterotoxoid, and tritotoxoid. This brings me to the crux of my views concerning the constitution of diphtheria poison.

In titrating and evaluating the diphtheria antitoxic serum I began with the simplest assumption, namely, that the poison was a simple uniform substance. In the formation of toxoids, therefore, I considered three possibilities:

1. That the affinity of the haptophore becomes increased;
2. That it remains the same, and
3. That it decreases.

Which of these possibilities will apply in any given case will, of course, depend upon the stereochemical circumstances, especially upon how far one functioning group is removed from the other. If, in what we must conceive to be a very large molecule, these groups are quite far apart, it may be assumed *a priori* that the destruction of the toxophore group will probably not exert a marked influence on the haptophore group. In other words, syntoxoids will be formed. If the two groups are nearer together a change in the affinities, either positively or negatively, can readily occur. As a matter of fact, the possibility of an increase or decrease of affinity as a result of this transformation into inert modifications has also been observed in connection with related subjects. Researches conducted by myself and Sachs have shown that in the formation of complementoid the hap-

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was the expression of a certain loss of combining groups. This, however, is merely apparent; in the second phase a greater excess of the poison (containing, as it does, more toxoid) is required to produce death than is the case with the hemitoxin. Bearing this consideration in mind it is easy to convince one's self that not a single one of the combining groups present has been lost and that the change which the poison has undergone was a quantitative one.

tophore group suffers a *decrease* in affinity. Complementoids, it will be remembered, result from the destruction of the zymotoxic group, the analogue of the toxophore group. Eisenberg and Volk by their discovery of proagglutinoids have shown that in the formation of agglutinoids an *increase* in affinity can take place.

Hence in diphtheria poison the possibility had to be considered that similar conditions obtain in toxoid transformation. In this case, however, it was remarkable that this toxoid formation did not always follow the same scheme, the poison, of course, always being thought of as a simple uniform substance. I was finally able to solve this problem in the following manner.

My earlier investigations had given me the impression that 1 I. E. (immune unit) should neutralize 200 fatal doses of a pure toxin, one consisting only of toxin molecules and therefore free from toxoids. I am quite ready to admit that I did not at that time furnish any absolute proof for this view. My first effort was therefore directed to a study concerning the correctness of the figure 200. I began by analyzing a large number of different toxins in the hope that sooner or later I would find an ideally pure toxin. I have already mentioned that the highest purity thus far obtained, a toxin obtained by Madsen, corresponds to only four-fifths purity,  $L_+$  containing 160 L. D. Nevertheless by means of the method of neutralization I was able to find poisons which fulfilled my requirements, at least in part. This was the case, for example, in my Poison No. 2 (see spectrum, Fig. 2). In this the  $L_0$  dose contained 84 L. D. The first third of the

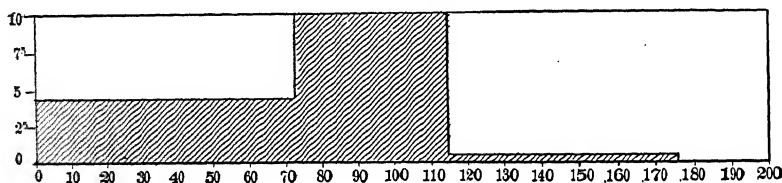
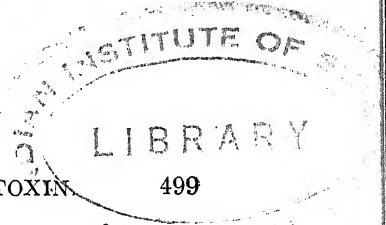


FIG. 2.

spectrum was taken up by a zone of hemitoxin not quite pure, i.e., each combining unit added  $\left(\frac{1}{200} \text{ I. E.}\right)$  decreased the toxicity by about  $\frac{1}{2}$  L. D. In the next zone, on the other hand, stretching from 72 to 115, each combining unit took away exactly 1 L. D. The spectrum is here reproduced. It shows the zones of hemitoxin, pure toxin, tritoxoid, and toxon very clearly.



Madsen, too, has described a poison "C," the constitution of which is very interesting because prototoxoid and pure toxin are distinctly marked off from one another. During the phase at which Madsen examined it the pure toxin zone occupied the zone 50 to 100 of the spectrum. Before the formation of tritotoxoid this zone may, however, have extended to 150.

From these observations we see that for certain portions of the spectrum (which lie in the middle and not at the commencement<sup>1</sup>) it has been possible to prove that  $\frac{1}{200}$  I. E. combines with exactly 1 L. D. This argues strongly in favor of the correctness of my assumed figure 200. In these zones of pure toxin only toxin molecules are neutralized and no toxoids.

Although it is rare to find zones of pure toxin in poisons which have been kept some time, it is extremely common, or even constant, to find in these older poisons zones in which  $\frac{1}{200}$  I. E. neutralizes exactly  $\frac{1}{2}$  L. D. Manifestly under these conditions equal parts of toxin and toxoid must always be neutralized; for this reason I have termed such a poison a hemitoxin. The following scheme represents such a changed poison:



FIG. 3.

It needs no further explanation to show that in this hemitoxin zone the affinity of toxin and toxoid to antitoxin has remained unchanged.

The entire process of toxoid formation takes place in two phases, as can readily be seen from the initial zones of suitable spectra (see Fig. 3). The pure toxin first changes into hemitoxin; in the second phase, however, the hemitoxin changes into pure toxoid, especially in the first part of the spectrum. This is illustrated by the following scheme:

<sup>1</sup> In the curve of ammonia-boric acid and of tetanolysin the maximum combining power always occupies the very first portions of the curve.

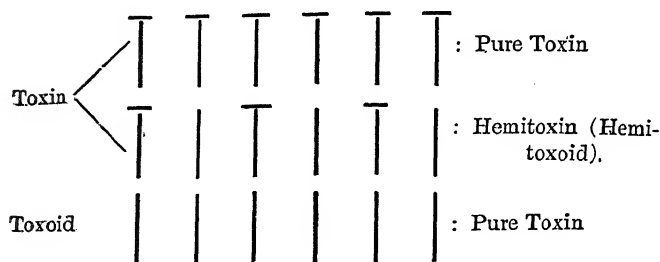


FIG. 4.

I must again emphasize that this sketch of the decomposition of the poison is not at all hypothetical, but merely the expression of the facts observed. The regular course in two phases points directly to the fact that the individual toxins are not simple uniform substances but are composed of two modifications present in equal amounts in the toxin solution and behaving differently on decomposition. One, the more unstable of the two, the  $\alpha$ -modification, decomposes rapidly and so gives rise to the stage of hemitoxin. The subsequent destruction of the more stable  $\beta$ -modification leads to pure toxoid. It is, of course, somewhat remarkable that exactly equal parts of two toxin modifications should develop in diphtheria bouillon. This is readily understood, however, if we remember that E. Fischer has made it extremely probable that the active groups of ferments (groups exhibiting a great similarity with the toxophore group) possess an asymmetrical constitution. If then in accordance with this we assume an asymmetrical constitution of the toxophore group, there will be nothing remarkable in the fact that the diphtheria bacilli produce both asymmetrical components simultaneously. Nor is it surprising that both are produced in equal amounts if we consider, for example, that optically inactive tartaric acid consists of equal parts of dextro and lævo tartaric acid. If optically active combinations (of which a large number can be made artificially) are produced in the retort, the rule holds that exactly the same number of molecules of the two components are produced by the reaction.

Ever since Pasteur showed that in the fermentation of tartaric acid by moulds the dextro tartaric acid is decomposed first, it has been found possible to demonstrate a similar behavior in numerous other instances; thus by the aid of moulds, yeasts, and bacteria it was found possible to isolate one of the optically active components from racemic

combinations. Looked at in this way the formation of hemitoxin is explained in very simple fashion.<sup>1</sup>

It can readily be shown that in the first stage of toxoid formation which leads to hemitoxin no change in affinity takes place, and this holds true also for all the toxoid formation, for if an increase in affinity occurred there could be no hemitoxin zone; a prototoxoid zone would again be followed by a zone of pure toxin. Conversely if there were a decrease in affinity a zone of pure toxin would precede the toxoid portion. The following scheme will serve to make these conditions clear:

These considerations at once show us that in the formation of toxoid no change in affinity can take place. As a matter of fact, however, the prototoxoid possesses a much stronger, and the tritotoxoid a much weaker, affinity than the toxin or hemitoxin occupying the central portion of the spectrum.

This we saw in our analysis of the poison mentioned above. We must, therefore, conclude that this difference is not produced by the formation of toxoid, but exists in the toxic bouillon from the beginning, the initial portion of toxin, which subsequently passes over into prototoxoid, already possessing a higher affinity for the antitoxin. The poison of diphtheria, for example, could be represented by the following rough diagram, in which the degree of affinity is expressed schematically by the length of the lines:

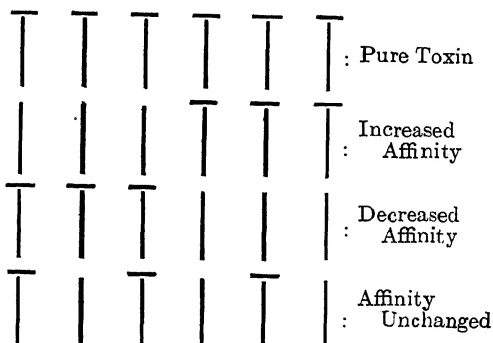


FIG 5.

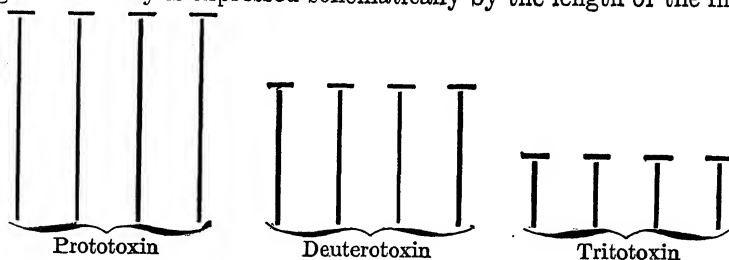


FIG. 6.

<sup>1</sup> See E. Fischer, *Zeitschr. f. physiol. Chemie*, Vol. 26.

Certain other considerations have convinced me of the plurality of the toxins. Chief of these is the behavior of the poisons on long standing. As is well known, poisons freshly produced rapidly deteriorate in toxicity until a point is reached beyond which the constants of titration, especially  $L_t$ , remain unchanged. Such "ripened" poisons are made use of in the official testing of diphtheria antitoxin, and we have therefore had abundant opportunity to convince ourselves that they remain constant.

From the standpoint of physical chemistry this fact (that the toxicity after a time becomes constant) could perhaps be ascribed to an equilibrium between toxin and toxoid. Such an equilibrium, however, is found only in reversible reactions, i.e., in chemical processes, which also proceed in the reverse direction. Toxoid formation, however, is not a reversible reaction; no one has yet discovered even a suggestion of a toxoid passing over into toxin. Another point which speaks against a condition of equilibrium is the fact that through artificial influences—heat, chemicals—any desired proportion of toxin and toxoid can be produced. Only one other explanation therefore remains, namely, that various toxins are present, of which some are more resistant, others less so.

I have thus presented in detail the reasons which led me to assume the existence of *preformed* varieties of toxins. As a result of my experiments I must emphatically deny the assumption that the phenomena observed by me in diphtheria poison are only the expression of a weak affinity between diphtheria toxin and antitoxin. I have demonstrated that the observed deviations can only be due to the admixture of toxoids with different affinity, and have further made it probable that these different degrees of affinity exist preformed in the toxin and do not arise with the formation of toxoid. It must, however, be distinctly understood that the points of view here laid down are not applicable to the relations between toxins and antitoxins in general. They apply only to diphtheria toxin and its antitoxin. The important researches of Arrhenius and Madsen on tetanolysin show that neutralization proceeds in an entirely different fashion when the two components possess a weak affinity for one another. The studies of these authors clearly indicate the errors in the interpretation of neutralization phenomena when dissociation is disregarded.

My results were obtained by the long and tedious experimental method. I can assure the reader that the experiments upon which all this is based, experiments carried out by my fellow workers (espe-



cially Geh.-Rath Dönitz and Dr. Morgenroth) and myself, have been most exact, and I venture to say that in medicine but few investigations exist which have been carried out with such precision and on such abundant material.

## II. Toxons.

Thus far we have dealt only with the true toxin portion of the diphtheria poison, and have entirely disregarded another constant secretory product of the diphtheria bacillus, namely, the *toxons*. On testing a diphtheria poison and determining the two limits,  $L_0$  and  $L_+$ , we should expect that the difference,  $L_+ - L = D$ , would correspond exactly to one lethal dose, provided the poison were a simple uniform substance. Thus if  $L$ , for example, contains  $a$  lethal doses these, according to our definition of  $L_0$ , will exactly be neutralized by 1 I. E. Assuming that the two substances have a strong affinity for each other, the addition of one L. D. would suffice to transform this neutral  $L_0$  mixture into  $L_+$ , i.e.,  $L_+$  should contain  $(a+1)$  L. D. and the difference,  $D$ , should equal 1. As a matter of fact, however, it was found that with the exception of one poison examined by me, the difference between  $L_+$  and  $L_0$  is much greater. In the poisons described in my first communications the difference  $D$  ranged from 5 to 50 L. D. At first, when I still held to the unitarian conception, I had interpreted these results as indicating the existence of a toxin derivative of very little toxicity and possessing less affinity than the toxin. For this reason I termed the derivative "epitoxoid." In my second communication, however, I abandoned this assumption, and stated that we were evidently dealing with a primary secretory product of the diphtheria bacilli—the "toxoid." The reasons which led me to this view will be presented in a moment. The toxoid possesses the same haptophore group as the toxin, but a weaker affinity for the antitoxin. The main difference is in the toxophore group, for even when given in large doses the toxoid does not produce death, but only paralyses which develop after a long incubation of fourteen days or more.<sup>1</sup>

Arrhenius and Madsen have doubted particularly the existence of

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<sup>1</sup> It may be remarked in passing that such additional or "by-poisons" with a long period of incubation are not limited to diphtheria bacilli. According to the observations of Sclavo on animals infected with anthrax it is highly probable that anthrax bacilli also produce poisons having a toxin-like action.

the toxons. According to them the long-drawn-out toxon zones are the expression of the incomplete combination of toxin and antitoxin, the neutralization of which they believe follows the ammonia-boric acid type. There are, however, a number of weighty reasons why this view cannot be accepted.

It was but natural at first to ascribe the toxon stage to phenomena such as Arrhenius and Madsen now have in view. It had already been noticed by others that often a considerable interval exists between  $L_{\dagger}$  and  $L_0$ . Knorr, in referring to this, had spoken of "un-neutralized poison residue." The assumption, however, that we are here dealing with the result of an incomplete neutralization is controverted by the analysis of a poison which I encountered during the course of my investigations. This was Poison No. 10 (of my series), whose  $L_0$  and  $L_{\dagger}$  values were very close together.  $L_0$  contained 27.5 and  $L_{\dagger}$  29.2 L. D. Hence  $D=1.7$  L. D., which is a close approach to the figure demanded by a simple diphtheria poison.

The following considerations will show that this value, 1.7, should be corrected so as to be still lower. The original calculations were based on my earlier assumption that toxins and toxoids are uniformly mixed. This however, has been superseded by the spectrum method of representing the neutralization of poisons. Experience has taught us that such deteriorated poisons usually consist of a small zone of hemitoxin and a more or less pronounced zone of tritotoxin-toxoid, in which as a rule nine toxoid equivalents fall on one toxin equivalent. Several times I have observed tritotoxin-toxoid zones containing  $\frac{1}{10}$  toxin, and Madsen also has described such a poison. As can be seen from our calculations given above, the theoretical change from  $L_0$  to  $L_{\dagger}$  is influenced solely by the tritotoxoid zone. If we therefore assume that our poison possessed a tritotoxin-toxoid portion whose strength was  $\frac{1}{10}$  (and this is extremely probable) we shall find that by a little calculation that the poison probably contained no toxon whatever. Very likely the tritotoxoid zone reached to the end (200) of the spectrum. On the assumption of a  $\frac{1}{10}$  tritotoxin-toxoid, if we multiply  $L_0$  by  $\frac{210}{200}$  we shall obtain  $L_{\dagger}=28.9$  L. D. This agrees very well with the figures obtained experimentally,  $L_{\dagger}=29.2$  L. D.

We may therefore very well assume that we were dealing with a poison free from toxon or one which contained only very small traces of toxon.

This fact is hard to reconcile with the theory of Arrhenius and Madsen, for if toxin and antitoxin neutralized each other like ammonia and boric acid, *all* poisons should show a long zone of incomplete neutralization.

The independent existence of the toxons is further corroborated by the fact that the toxon zone varies enormously in different speci-

mens of poison. In one it may amount to about one-fifth of the toxin portion, in another I have seen equal parts of toxon and toxin. Dreyer and Madsen in fact have recently described a poison which contained three times as much toxon as toxin. According to our present experiences, therefore, the amount of toxon calculated on the toxin can vary from 0 per cent to 300 per cent. Hence I find it impossible to assume that we are dealing with neutralization phenomena such as are observed with ammonia and boric acid, for such neutralizations would show at least some agreement.

This still left undecided whether the toxon is a primary bacillary secretion or a secondary modification of the toxin. A study of the development of one poison finally gave me the clue to this. This was poison V, whose constitution has been described in the *Deutsche med. Wochenschrift* 1898. It will be recalled that this poison possessed the following limits in the second phase:

$$L_0=0.125; L_{\dagger}=0.26; L. D.=0.003.$$

During the course of three weeks Geheimrath Dönitz made continuous determinations of  $L_0$  and  $L_{\dagger}$ , using very uniform animal material. The protocol of this experiment is reproduced in full because the precision of the methods will thereby also be exhibited (see table on page 506).

From the table we see that in the course of three weeks  $L_0$  has increased from 0.15 to 0.20. After this an insignificant increase brought this to 0.21; from then on  $L_0$  remained constant. During this time the  $L_{\dagger}$  dose (0.26) had suffered no change whatever, for on the 16th of July a mixture of 0.25 poison + 1 I. E. killed in six days and 0.275 + 1 I. E. in three days.  $L_{\dagger}$ , which according to our definition is the mixture that will just kill on the fifth day, must have been about midway between these two values, a little over 0.26. This agrees very well with the value obtained in the beginning. To repeat, during the course of this stage  $L_{\dagger}$  has remained constant, but  $L_0$  has increased considerably (from 0.125 to 0.21).

This fact is easily explained. The toxin portion has remained absolutely unchanged in its end zone, as can at once be seen from the constancy of the  $L_{\dagger}$  dose. On the other hand in the toxon portion, which is expressed by the difference between  $L_{\dagger}$  and  $L_0$ , 80 toxon equivalents out of 100 have apparently disappeared. This eliminates the possibility of a transformation of toxin into toxon, for if that assumption were correct one would expect that on allow-

ing the bouillon to stand, the toxin zone would decrease and the toxon zone become considerably greater. In this case, however, we see that the toxin zone remains constant while the toxon zone is reduced to one-fifth.<sup>1</sup>

DETERMINATION OF  $L_0$  DOSE.

Amount of Poison cc.	Guinea-pigs are Injected with 1 I. E. + Varying Amounts of Poison.						
	June.			July			
	21	25	29	1	4	6	10
0.125	—	0	—	—	—	—	—
0.1275	faint trace	almost 0	—	—	—	—	—
0.13	—	—	—	—	—	—	—
0.14	—	—	slight but distinct	—	—	—	—
0.15	—	—	—	just neutral	—	—	—
0.16	—	—	—	slight but distinct	—	—	—
0.17	—	—	—	—	little	slight	—
0.18	—	—	—	—	—	—	—
0.19	—	—	—	—	more	slight œdema	—
0.2	—	—	—	—	—	more œdema	almost neutral
0.215	—	—	—	—	—	more œdema	some œdema
0.23	—	—	—	—	—	—	marked œdema

"Faint trace," "slight," etc., denote the degree of infiltration.

It is difficult to say *a priori* what has become of the toxon which has disappeared. On account of certain facts which I shall mention later, I have assumed that we are here dealing with the formation of an analogue of toxoid, namely, a substance which I term "toxonoid." I conceive this to be a toxon in which the toxophore group has become modified.

<sup>1</sup> The entire course of the decomposition, in which from day to day we could observe the toxon becoming weaker and weaker speaks against the possibility (in itself very remote) that the varying composition of the bouillon is responsible for the variation in the number of toxons in the individual poisons. In the poison here described the decomposition has taken place in the same bouillon and in so short a time that very great alterations in the bouillon appear to be excluded.

Another fundamental difference, one which in my opinion argues in favor of the individuality of toxin and toxon, consists in the different action of the two constituents. The action of diphtheria toxin, as is well known, is such that the animals die with symptoms of hydrothorax, ascites, congestion of the suprarenals, necrosis of the skin. Somewhat smaller doses kill guinea-pigs in from six to seven days, the animals showing ulceration and extensive necrosis. Still smaller doses,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ ,  $\frac{1}{16}$  L. D., no longer produce death, but regularly cause necroses which are surrounded by an extensive area of total loss of hair. Small fractions of the fatal dose always produce emaciation of the animals. In contrast to this, the toxon, i.e. a serum-poison mixture in which only the toxin fraction is completely neutralized, never kills animals acutely, even in high doses. The inflammatory properties may be entirely absent in small doses, while in large doses they are present to only a slight degree. The oedema disappears completely in the course of a few days, there are no necroses, and the loss of hair, if it occurs at all, is only partial. On the other hand the paralyzes are very characteristic, and these appear at any time between the fourteenth and twentieth day, depending upon the dose, usually in the third week. Frequently the animals do not show even a trace of local reaction and maintain their weight; then suddenly they are attacked with the paralyzes and may die from these within a few days. I have never seen such a result in animals inoculated with a pure diphtheria poison. Now and then a guinea-pig was observed which showed these paralytic phenomena. It was usually one that had received a considerable fraction of the L. D. Invariably it showed extensive necroses, was generally very sick from the beginning, and had suffered considerable loss of weight. In view of the slight amount of toxon which I found in these poisons, such animals were evidently supersensitive to the toxon.

Dreyer and Madsen have succeeded in differentiating toxin and toxon qualitatively, as follows: They found that mixtures of a diphtheria poison and antitoxin in which the limit of complete toxin neutralization was nearly approached, exerted only toxon effects when given in small doses. If, however, the mixture was increased tenfold, death was brought about by the toxin. This is readily explained. The determination of toxon by means of 1 I. E. naturally cannot be absolutely exact, for a small residue of toxin, e.g.  $\frac{1}{10}$  L. D., can readily escape observation. If, however, a sufficiently large multiple of this mixture, e.g. ten times the original quantity, is

injected, this will now contain  $10/10$  L. D. unneutralized. If now the amount of antitoxin was also somewhat increased, Dreyer and Madsen found that even with this multiple amount only toxon effects were observed, the toxin now being completely neutralized and only toxon remaining free.

Dreyer and Madsen<sup>1</sup> thereupon subjected this same poison to a thorough study, using rabbits for the purpose. They found if 0.6 cc. poison was mixed with 1 I. E., that this mixture, which represents the  $L_0$  dose for guinea-pigs, is still highly toxic for rabbits. In order to render this dose of poison completely innocuous for rabbits it is necessary to add more antitoxin, in this case  $\frac{240}{200}$  I. E. The statements concerning the behavior of mixtures between these two limits are also of considerable importance. A mixture of 0.6 cc. poison +  $\frac{210}{200}$  I. E. injected into a rabbit causes death on the twenty-second day with paralytic symptoms. The incubation period is sixteen days. Even a mixture of  $\frac{232}{200}$  I. E. with the same amount of poison caused paralyse, which appeared on the sixteenth day and continued for several weeks. This behavior is so important for our view concerning the existence of different poisons that I must enter a little more fully into the subject. According to our definition of the  $L_0$  dose, mixtures like the one containing  $\frac{232}{200}$  I. E., and therefore possessing a considerable excess of antitoxin, are absolutely innocuous for guinea-pigs and can be injected in any quantity. In virtue of the excess of antitoxin such mixtures suffice to passively immunize the animal and to protect it, provided suitable doses have been injected, against diphtheria poison and diphtheria bacilli. If then such mixtures are still toxic for rabbits only one possibility remains, namely, that the diphtheria poison in question contains a substance which is non-toxic for guinea-pigs but toxic for rabbits. This substance I term toxonoid.<sup>2</sup>

<sup>1</sup> See also my article in Münch. med. Wochensch. 1903, Nos. 33, 34.

<sup>2</sup> At the outset of my investigations I made entirely similar observations. My very extensive but unpublished studies made at that time convinced me that this property is not common to all diphtheria poisons, for I also found some in which the  $L_0$  dose was exactly the same in rabbits and in guinea-pigs. This fact furthermore refutes the assumption that the phenomenon described

So far as the behavior of partially neutralized mixtures is concerned, the observations of these authors show that mixtures which exert only toxon effects on guinea-pigs produce death in rabbits with symptoms of diphtheria poisoning. I believe that all these phenomena are best explained by the assumption that there are at least three different varieties of poisons, and that these possess different affinities and different actions. These poisons are:

1. Toxin, possessing the highest affinity, kills rabbits and guinea-pigs acutely, but is more toxic for the former.
2. Toxon, killing rabbits acutely and guinea-pigs with symptoms of paralysis.
3. Toxonoid, producing paralyses in rabbits, non-toxic for guinea-pigs.

The fact that all three poisons act more strongly on rabbits than on guinea-pigs is explained by the absolute higher susceptibility of the former.

Dreyer and Madsen have recently described a diphtheria poison in which toxoid effects could be demonstrated even on the injection of sublethal doses of the pure poison. This behavior is at once understood if we study the constants of this poison as they were determined by these authors, for whereas in the other poisons examined there were 33 toxon equivalents to 167 toxin equivalents (toxon:toxin = 1:5), in this poison the proportion was just the reverse, there being three times as much toxon as toxin. No wonder therefore that with the toxon fifteen times more concentrated even sublethal doses of the pure poison should suffice to make toxon effects evident.

In view of the high theoretical significance which attaches to the poison described by Dreyer and Madsen, I cannot refrain from giving briefly my conception of its constitution. The authors have represented the poison in the form of a curve, one which at first sight seemed rather strange to me. As soon, however, as I transformed their graphic representation into a spectrum by the aid of their figures, the constitution of the poison was found to agree very well with other well-known diphtheria poisons. The only difference is the very

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is due to an incomplete neutralization, such as Arrhenius and Madsen, for example, have demonstrated in the case of boric acid and ammonia, and in the union of tetanolylin with its antitoxin. If that were the case one would expect to see the phenomenon in all diphtheria poisons in equal degree, and this is not the case.

large content of toxon. The spectrum, which corresponds to the curve obtained by the authors, is here reproduced (Fig. 3, Phase II).

From this we see that a zone of hemitoxin in the beginning of the spectrum is followed by a zone of almost pure toxin, and this in turn by a zone of tritotoxin-toxoid. Then comes the very long toxin fraction.

To one employing this mode of representation, such a spectrum not only pictures the present constitution of the poison but also frequently permits him to reconstruct its previous constitution. In this case, for example, it was possible to do so with the aid of several statements by the authors concerning earlier and later stages. According to these figures I would assume that in the first phase the poison contained a pure toxin in the initial zone. In the second phase, the period at which the poison was studied by Dreyer and Madsen, this had become transformed into hemitoxin. In the third phase it may become pure prototoxoid. A fourth phase would then show the transformation of the pure toxin in the above spectra into hemitoxin and the poison would then have reached the point which we have so frequently observed in other poisons. The spectra of these various phases is as follows (Fig. 7):

I shall now present the figures which Madsen and Dreyer obtained when they started with double the  $L_0$  dose (0.1 cc. poison). In the first phase, their statement that the lethal dose was 0.0015 cc. shows that 0.1 cc. poison contains 66 L. D. Calculation from the spectrum gives 65 L. D.

The second phase, of course, agrees entirely with the statements of the authors, since the spectrum was constructed according to these.

In the third phase the formation of the prototoxoid zone from the previous zone of hemitoxin is readily seen from a second neutralization test, one made with normal horse antitoxin.

In phase IV the lethal dose had risen to 0.0027, corresponding to 37 L. D. in 0.1 cc. Calculating this from my spectrum I obtain 35 L. D., which is but 2 L. D. smaller than would correspond to the final stage. Perhaps this stage had been nearly but not yet completely attained. It is probable that if the examination had been made a little later the figure would have been exactly 35.

The figures obtained from my reconstructed spectra harmonize so well with those obtained experimentally by the authors that it seems almost impossible to doubt the correctness of my assumptions concerning the constitution of the poison and the process of its trans-



formation. This proves that in this poison the toxin zone behaved exactly the same in its transformation as it did in the other diphtheria poisons examined.

I believe it will be seen from my explanations that my mode of procedure in the study of diphtheria poison has been exceedingly

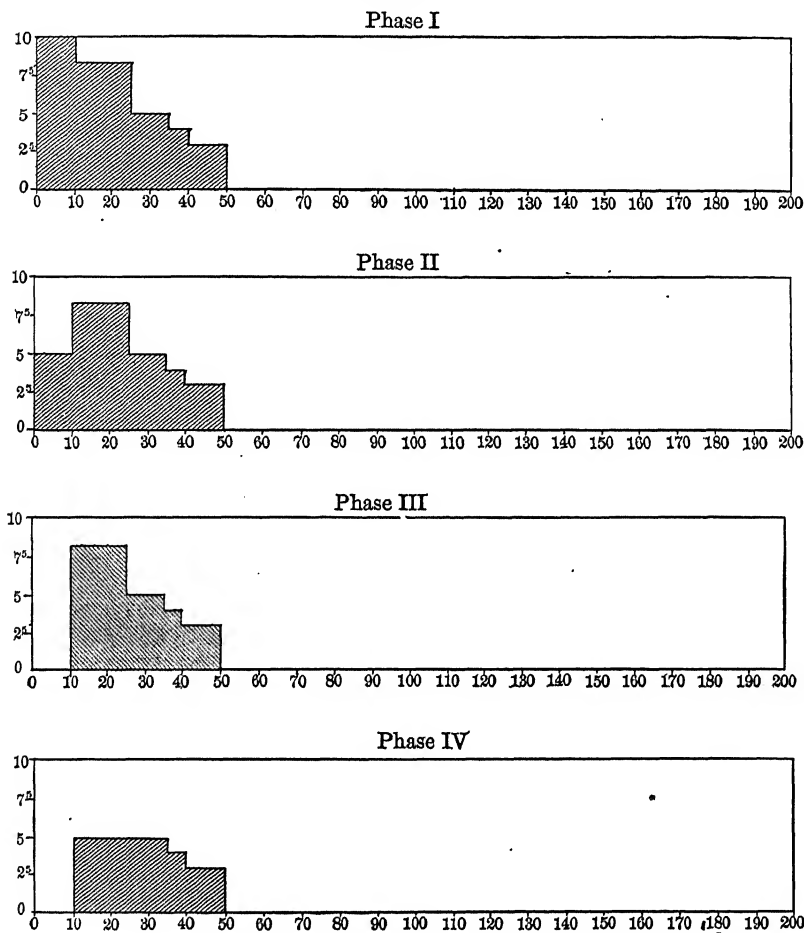


FIG. 7.

careful, and that the objections raised against my results do not apply. I must therefore continue to maintain my original standpoint, and deem it well therefore to once more define my views concerning the poison of diphtheria.

1. The diphtheria bacillus produces several kinds of poisons, especially toxins and toxons.

2. The affinity of diphtheria toxin to the antitoxin is very great.

3. The deviations from a straight line as they manifest themselves in the graphic representation of the neutralization of the poison cannot be explained by the assumption of a single poison possessing a weak affinity. They are rather the expression of the fact that the poison bouillon contains admixtures of various kinds of substances of a toxoid character.

4. The varied affinity of the toxoids cannot be explained by the assumption that a simple toxin when transformed into toxoid suffers a change in affinity either positively or negatively. Rather does this indicate that the toxic bouillon contains, preformed, various toxins of different affinities.

5. There is no change in the haptophore group in the formation of toxoid.

6. The absolute number of combining units contained in the immune unit or in the  $L_0$  dose of poison is 200.<sup>1</sup>

I have finished. If the results of the first encounter of two such different methods of study as the mathematico-physical and the biological have not shown complete agreement we should not be at all surprised. The natural aim of physical chemistry must always be

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<sup>1</sup> Bordet has recently attempted to explain the toxon phenomena by the assumption that the toxin molecule can combine with antitoxin in varying proportions. One would accordingly have to assume that the toxin molecule contains several haptophore groups. The complete occupation of these groups causes the toxicity to be entirely lost, whereas partial saturation causes a decrease in toxicity. That is to say, amounts of antitoxin which do not completely neutralize the toxin would weaken it in such fashion that it would exert a different action. It is strange that so eminent an investigator as Bordet should not have attempted to convince himself of the correctness of this hypothesis by means of the experiment. He would then have found that the facts are irreconcilable with such an assumption. We have shown at great length that the toxon actions are nothing less than constant phenomena and have called attention to the great extent of the quantitative variations (0-300). If one were to follow Bordet it would then be necessary to assume an enormous multiplicity of haptophore groups in the toxin molecules, and this would lead to a hypothesis far more complicated than mine, although the latter harmonizes all the experimental results. In support of his conception Bordet refers to experiments with complement and anticomplement. I must say, however, that in these we are dealing with such complicated relations that it is not permissible to apply the conclusions drawn from them to the far simpler relations existing between toxin and antitoxin.

to introduce as few factors as possible for purposes of calculation, whereas biological analysis always seeks to pay due regard to the wonderful multiplicity of organic matter. However, I believe that these two methods can readily be combined and that this will be very desirable. The biologist will have to content himself in so far yielding to the economy of the mathematical view that he restricts his assumptions to the smallest possible number. The physical chemist, on the other hand, cannot escape the obligation of paying due heed to this minimal multiplicity, the result of experimental research. Naturally the problem is thus made extremely difficult, so that success will require that the greatest authorities in physical chemistry work hand in hand with the best biological talent. For this reason I regard it as a great gain to science that so eminent a leader as Svante Arrhenius is taking a lively interest in our work, and has joined hands with my friend and pupil, Thorvald Madsen.

## A REPLY TO THE LATEST ATTACK OF GRUBER.

By PAUL EHRLICH.

IN a domain that is open to experimental investigation it is neither easy nor without danger for one to express criticism merely as a result of literary studies.

This is especially true in that most difficult field in the entire study of immunity, namely, the subject of toxins. Only one who has devoted years of unprejudiced study at the laboratory table to this subject and gathered a host of observations and experiences will be in a position to orientate himself in the confused mass of true and false statements contained in the literature. The outsider will find it very difficult to correctly analyze all this material. Hence it is all the more remarkable that Gruber<sup>2</sup> should choose the subject of toxins for the main portion of his attack upon me, for according to his own admissions that is the field which he knows merely from literary studies. Against such critics I am in the unpleasant position of a man who is compelled to discuss colors with the blind. Nevertheless I cannot well escape the thankless task of replying, at least to the main points in Gruber's polemic, for it is indisputable that this attack, addressed chiefly to those without special training in this field, is capable of causing wide-spread confusion, owing to its positive tone and its severity.

Gruber's first important error lies in the assumption that a controversion of the plurality of poisons, to which I hold, signifies the downfall of the side-chain theory without further ado. The side-chain theory, however, proceeds from the assumption that the toxin-

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<sup>1</sup> Reprinted from the Münch. med. Wochensch. 1903, Nos. 33 and 34.

<sup>2</sup> M. Gruber and Cl. v. Pirquet, Toxin und Antitoxin, Münch. med. Wochensch. 1903, Nos. 28 and 29.

like poisons are characterized by a haptophore and a toxophore group, of which only the former effects the anchoring of the toxin. Practically therefore only this group is important for the production of antitoxins. This view is only the logical consequence of the fact that on long standing the poison bouillon undergoes modifications, resulting in the production of what I term toxoids. These substances are characterized by this, that the haptophore group has remained intact, while the toxophore group, depending on circumstances, has suffered partial or complete modification. Not infrequently it can be shown that the formation of toxoid is quantitative, the combining power of the toxic bouillon being unchanged despite a considerable loss of toxicity.

Gruber, by means of certain calculations, appears to question this fact; he refers exclusively to my very earliest publications in which, naturally, the evidence was still incomplete. It would have been better if Gruber had studied instead my later publications, for then he could easily have convinced himself that my statement is entirely correct. I shall mention but one of my poisons<sup>1</sup> as an example. In this the L dose was originally 0.25 cc., the lethal dose 0.0025 cc. At the end of the investigation  $L_+$  had increased to 0.26 cc., the lethal dose, however, to 0.004 cc. The number of lethal doses, therefore, in approximately the same amount of  $L_+$  had been reduced from 100 to 65. Madsen<sup>2</sup> describes a poison in which the neutralizing power remained constant during the course of two years, while the toxicity was reduced one-half, from 0.02 to 0.04. Furthermore Arrhenius and Madsen, in their most recent work<sup>3</sup> describe the toxoid modification of a tetanus toxin. These consist in the fact that the combining power remains intact while the toxicity is decreased to one-sixth. It is seen therefore that the doubt thrown upon my quantitative statements is due entirely to a disregard of readily accessible facts. This quantitative transformation constitutes a somewhat annoying fact for Gruber, and he therefore seeks to explain it as follows:

"Imagine, if you will, that  $\frac{9}{10}$  of the toxin molecules present are changed into toxoids, the minimal lethal dose will then be increased

<sup>1</sup> Described in *Deutsche med. Wochens.* 1898, No. 38.

<sup>2</sup> *Annales de l'Institut Pasteur.*, T. 13, 1899.

<sup>3</sup> S. Arrhenius and Th. Madsen, *Physical Chemistry applied to Toxins and Antitoxins*, Festschrift ved. indvielsen af Statens Serum Institut, Kopenhagen, 1902; German in *Zeitsch. für physiol. Chem.* 1903.

tenfold whereas the  $L_0$  value will remain unchanged; this is Ehrlich's hypothesis. If  $\frac{9}{10}$  the toxin molecules had lost their toxicity, without there being any formation of toxoids capable of combining with antitoxin, the  $L_0$  value would be increased ten times. If, however, simultaneously with the loss of  $\frac{9}{10}$  the toxicity, the fluid were to lose  $\frac{9}{10}$  the reaction rapidity for antitoxin, so that the constant of the reaction would be decreased  $\frac{9}{10}$ , it would be found that the  $L_0$  value would manifest itself unchanged."

Gruber would have done better to have made some of these comparatively simple experiments himself than to advance such an untenable assumption. We are here dealing with experiments which constitute, in fact, the very beginning of the technique of testing poisons. Thus, when in 1897<sup>1</sup> I formulated the law that the combination of poison and antibody takes place more rapidly in concentrated solutions than in weak solutions, it was as the result of just such studies made on diphtheria and tetanus toxin. In these studies I convinced myself that the affinity between diphtheria antitoxin and diphtheria toxin is far greater than that between tetanus antitoxin and tetanus toxin. The union of diphtheria toxin and its antitoxin is effected very quickly, so that at the end of five to ten minutes one may be sure that complete union has taken place. It is entirely immaterial whether one is dealing with fresh poisons or with poisons poor or rich in toxoids. I shall here reproduce an experiment which I have recently made because Danysz<sup>2</sup> insisted that the neutralizing power of the diphtheria poison changes when the poison is allowed to stand for some time.

The experiment was performed with the standard serum and standard toxin used in the official standardization. Both substances had therefore been very accurately titrated. The mixture was allowed to stand fifteen minutes and twenty-four hours and the result showed that in this time not the least change had taken place in the constant. In the experiments of Danysz, therefore, some error has probably crept in. In any event there is no change in the reaction time on the decrease of toxicity of the diphtheria toxin.

Guinea-pig I receives 1 I. E. serum + 0.78 cc. poison ( $L_+$ ) fifteen minutes after mixing. It dies on the fourth day.

Guinea-pig II receives the same mixture twenty-four hours after mixing. It dies on the fourth day.

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<sup>1</sup> Die Werthbemessung des Diphtherieheilserums, Jena, 1897.

<sup>2</sup> Annales de l'Institut Pasteur 1902.

Guinea-pig III receives 0.8 cc. poison, otherwise same as I. It dies in three and one-half days.

Guinea-pig IV receives 0.8 cc. poison, otherwise same as II. It dies in three and one-half days.

Another thing which is entirely irreconcilable with Gruber's assumption is the fact that there exist prototoxoids, i.e., toxoids which possess a higher affinity for the antitoxin than the toxin itself does. The existence of these was first pointed out by me and has since been confirmed by Madsen and also by Arrhenius. The existence of the prototoxoids becomes clearly manifest by the fact that one can add a certain quantity of antitoxin to the toxin solution without affecting the toxicity in the slightest degree.

Mention must also be made of the fact that similar phenomena have been observed in a large number of other poisons. It will suffice here if I remind the reader that toxoid changes have been observed in ricin (Jacoby), abrin (Römer), staphylo toxin (Wechsberg, Neisser), cobra venom (Meyers, Flexner). Furthermore Morgenroth and I showed that in complement also there is a destruction of the real active portion, the zymotoxic group, while the haptophore group remains intact. The existence of complementoids has been demonstrated decisively by Sachs and myself,<sup>1</sup> although Gruber had termed them "merely fervent wishes floating about in the serum."

Furthermore it will be remembered that similar phenomena are observed in the agglutinins and coagulins (precipitins), the haptophore group of the agglutinin or the precipitin remaining intact, while the agglutinophore group is destroyed. This phenomenon was first pointed out in the excellent study made by Eisenberg and Volk in Paltauf's laboratory. Since that time a large mass of literature has grown up around this subject so that now there is not the least doubt concerning the existence of these substances, which normally occur in the form of proagglutinoids. A recent study by Korschun<sup>2</sup> makes it probable that something similar to this occurs in ferments, particularly in rennin. In all these various cases it seems to be the rule that the real functioning group is far more labile than the one which effects combination, namely, the haptophore group. Hence I believe that the formation of such

<sup>1</sup> See page 209.

<sup>2</sup> Zeitsch. f. physiol. Chemie, Bd. 37, 1903.

modifications must be classed with the positively demonstrated facts in medicine.

It is entirely incomprehensible how Gruber could believe that the possible controversion of the plurality of poisons assumed by me denotes the downfall of the entire side-chain theory.<sup>1</sup>

How false such a conclusion is can be seen from the fact that when I devised the side-chain theory I believed the diphtheria poison to be a simple substance. My later studies, however, convinced me that the poison consists of several modifications: prototoxin, deuterotoxin, tritotoxin, and toxon. It can easily be seen from my publications, however, that I ascribe the same combining group to all of these; they differ merely in their toxophore groups. In the production of diphtheria antitoxin all of these modifications act in exactly the same way. It shows a deplorable lack of comprehension, therefore, when Gruber says that the refutation of the plurality of toxins will "give this side-chain-theory spook its quietus."

However, let us see what proofs Gruber advances against the plurality of the poisons. On a previous occasion when Gruber brought forward these same arguments I allowed them to pass without specially controverting them, for I felt that his faulty mode of reasoning would at once be apparent to the specialist. Now that Gruber, however, returns to this subject I think it may be well to discuss the facts somewhat in detail.

In the majority of poisons it is probably a fact that the toxicity depends upon the animal species, a certain poison being more toxic for one species than for another. In chemically definite poisons, alkaloids, etc., this behavior is usually a constant one, so that in text-books on toxicology the fatal doses per kilo of body weight

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<sup>1</sup> Arrhenius and Madsen (l. c.) in their very interesting study have questioned whether the phenomena of neutralization, which I described and referred to a plurality of poisons, are due to a difference in the poisons or whether, as they think probable, they are merely the expression of a neutralization between two substances of weak affinities. For the present I shall merely point out that my own statements refer only to diphtheria toxin, which possesses a much higher affinity for the antitoxin than does tetanus toxin. The investigations of these esteemed authors have disclosed one source of error which could easily creep into neutralization experiments. Nevertheless I believe that their conception does not apply to the toxin of diphtheria which I have studied so closely. I shall go into these questions more fully elsewhere, and hope then to show that the standpoint maintained by me is entirely correct.



are usually given for various animal species. In the beginning it was thought that the same conditions held true for the bacterial poisons and several such scales of toxicity were given out by high authorities. As soon, however, as different toxin solutions of the same species were examined, e.g. diphtheria toxins obtained from different cultures or in different laboratories, it was found that, unlike the alkaloids, the scale of toxicity was a variable one. In the case of one poison, for example, I found that a guinea-pig of 250 grammes was uniformly killed by a dose of 0.00375-0.004 cc., and a rabbit of 1800 grammes by a dose of 0.009 cc. This corresponds to a ratio of 1:2.2-2.4. In another poison the figures were 0.003 for guinea-pigs and 0.004 for rabbits, corresponding to a proportion of 1:1.3. This showed that in two different poisons the susceptibility of rabbits varied more than half.

The conditions, however, are far more interesting and instructive in the case of tetanus poison. For a long time a controversy existed between v. Behring and Tizzoni. The former stated that tetanus poisons act 150 times weaker on rabbits than on mice, whereas Tizzoni declared that a poison prepared by him was just as toxic for rabbits as for mice. From the papers of these authors it is certain that the two poisons when tested on mice were identical. A definite amount of either poison—for example, a single fatal dose for mice—was neutralized by the same quantity of antitoxin. So far as mice were concerned, therefore, the two poisons were identical. As soon as the poisons were tested on rabbits, however, the above-mentioned enormous difference in toxicity becomes apparent. This at once shows that these two poisons cannot possibly be identical. Wherein, then, does the difference consist? We have seen that the two poisons are neutralized by the same antitoxin, and that furthermore immunization with one of the poisons is followed by the production of an antitoxin, which acts also on the other poison. From this it follows that the haptophore group must be the same in both. Hence we must be dealing with a difference in the toxophore group, v. Behring's poison possessing a toxophore group which is highly virulent for mice and only slightly so for rabbits, whereas Tizzoni's poison contains a group which acts equally on both animals. This difference would be very like that which I have demonstrated in the case of diphtheria toxin and toxon. One might, however, think of an entirely different explanation, namely, that the strain of bacteria with which Tizzoni worked secreted an entirely

different kind of poison than the Marburg culture. But this proved not to be the case, for v. Behring demonstrated that his tetanus poison when injected into rabbits in large quantities suffers a considerable diminution in toxicity. On testing the properties of the poison contained in the serum of the poisoned animals he found that this residual poison possessed the same constants as Tizzoni's poison. From this it follows that v. Behring's poison contained also a certain proportion of the Tizzoni variety. The Marburg culture must therefore have produced two varieties of poison at the same time. Naturally by mixing the two poisons one can obtain new poisons which, while they manifest the same action on mice, will have any desired relative toxicity for rabbits; this, of course, within certain limits. If one were to take the time and trouble to examine a large number of native poisons from different laboratories, corresponding differences between them would probably be encountered.

If we recollect that various specimens of the chemically simple poisons manifest the same relative toxicity on different animals, and then consider the behavior of tetanus toxins as just described, we shall conclude that bacterial poisons of different origin, which manifest a variation in their relative toxicity, are not of simple constitution, but are made up of several different constituents. It shows very little knowledge of the subject therefore when Gruber says: "v. Behring shows that two toxin solutions, which in a given unit of volume contain equal  $\dagger$  Ms., i.e., whose unit of volume kills a like number of grammes of mouse in four days, may have an entirely different content of  $\dagger$  rabbit,  $\dagger$  pigeon,  $\dagger$  goat, and  $\dagger$  horse. This at once disposes of Ehrlich's conclusions." It is just such phenomena which argue in favor of the plurality of poisons; they do not speak against it.

Gruber bases another of his objections on the interesting observations made by Madsen and Dreyer on toxons (*Zeitsch. f. Hygiene*, Vol. 37, page 251). In his dictatorial manner he says that "these observations demonstrate conclusively that Ehrlich's method of analyzing toxins is absolutely useless. Only a person ignorant of chemistry could maintain that the different results in guinea-pigs and in rabbits are sufficiently explained by the different susceptibility of the animals to the toxins."

To begin, Gruber's premise is absolutely misleading, when he says:

"But if the poison is neutralized it will be without effect even

on the most susceptible animals. Let us imagine, for example, a mixture of sulphuric and acetic acids, neutralized by the gradual addition of baryta water. Once all the sulphuric acid is neutralized, even the most sensitive reagent to free strong mineral acids will be unable to detect any trace of it."

Let us see just what Gruber means by this comparison. The sulphuric acid corresponds to the toxin; the antitoxin is represented by the alkali. In accordance with the comparison the receptors of the cells are represented in the animal body by the alkali of the tissues. If now we inject an animal with sulphuric acid previously neutralized with ammonia, i.e., a solution of ammonium sulphate, it will depend mainly on the affinity of the tissue alkali, whether or not the neutral ammonium sulphate will be decomposed and sulphuric acid allowed to enter the tissues, ammonia being set free. If we assume, for instance, that the tissue alkali is comparable to a strong base like sodium hydroxid or barium oxid, the ammonia introduced in combination with the sulphuric acid will be absolutely unable to prevent the poisoning; the weak base will be forced out of the salt and replaced by the stronger base. In general we must assume that the antitoxin possesses a higher affinity to the toxin than do the tissue receptors, for only on this assumption can we explain the protective action of the antitoxin. Numerous phenomena, however, indicate that the affinity of the tissue receptors can become increased. I had reached these conclusions long before the publication of my theory, which as many know I formulated years before it was published. The cause of this long delay was the phenomenon of hypersusceptibility, i.e., the peculiar fact that immunized animals, despite a colossal excess of antitoxin, succumb to the action of the poison. The first light on this subject was the study of Dönitz, in which it was shown that the poison shortly after its union with the tissues is but loosely bound. In the course of a few hours the union becomes firmer and firmer so that after a certain time, which may vary from a few minutes to six hours, according to the dose, the poison can no longer be abstracted from the tissues by the antitoxin. This fact seemed to indicate that under the influence of the poisoning the affinity of the tissue receptors gradually becomes increased and that when a certain point is reached a cure by means of antitoxin is impossible. This, however, furnished me with an explanation of hypersusceptibility and removed the obstacle which had kept me from publishing my theory.

I should also like to mention that Kretz,<sup>1</sup> many years later and entirely independent of me, reached exactly the same conclusions as I had. His very interesting study was based on experiments with diphtheria-immune horses. Following his usual tactics, Gruber will, of course, draw the conclusion that the increase in the tissues affinity, since it agrees with my theory, cannot really occur, and he will therefore regard the entire subject as utterly fallacious and best not discussed. The unprejudiced observer, however, need hardly be told that it is impossible for chemical groups attached to living protoplasm to maintain their affinity unchanged as though they were made of stone; especially is this true if we consider the varying function of the protoplasm.

Let us take anilin as an example, and determine the combining heat of the  $\text{NH}_2$  group for a certain acid. We shall then find that nearly all substitutions of the benzol nucleus, as, for instance, the introduction of an amido group, a nitro group, a sulfo group, etc., markedly change the affinity either positively or negatively. Thus even the introduction of what is conceivably the most indifferent group, the methyl radical causes a distinct and marked diminution of the combining heat. Under these circumstances any one who thinks chemically would consider it peculiar if a change in the affinity of the cell constituents were to be regarded as something absolutely inconceivable and beyond the pale of discussion.

Since Gruber has given only that part of Madsen and Dreyer's experiments which fits into his polemic, it will be necessary for me to supplement this with some additional data from their study.

These authors employed a diphtheria poison of which the fatal dose for a guinea-pig of 250 grammes was 0.009, and for rabbits of 1200-1600 grammes, 0.0076. Calculated per kilo this shows that the rabbits were about six times as susceptible as guinea-pigs. The  $L_0$  dose, i.e., that amount of poison, which is just completely neutralized by one immune unit, was 0.6 cc. for guinea-pigs. Right here I must emphasize that the  $L_0$  dose, as I conceive it, refers exclusively to guinea-pigs, since according to my experiences this is the only animal in which, thanks to the peculiar susceptibility, the constants of the poison can accurately be determined. In the serum mixture  $L_0$  all the constituents of the poison, toxin, and toxon are completely neutralized, so that not only the single amount but also

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<sup>1</sup> Zeitsch. f. Heilk., Vol. 23, 1902.

high multiples of this can be injected into guinea-pigs without causing a trace of local or general reaction. If the same amount of poison, 0.6 cc., was mixed with  $\frac{167}{200}$  I. E. instead of with one I. E. it was found that the toxin fraction had practically been completely neutralized, leaving only the toxons, characterized by the development of paralyses. Just in this poison Madsen and Dreyer have shown that the difference between toxin and toxon is qualitative and not quantitative. They found that mixtures of poison and antitoxin, which were near the limit of toxin neutralization, showed only toxon action when given in small doses, whereas when the mixture was increased tenfold, death occurred from toxin.<sup>1</sup>

*If, however, the quantity of antitoxin was also slightly increased, even the tenfold multiple showed only toxon action.* From these data we see that the poison consisted of about 167 units toxin-toxoid and 33 units toxon.

This same poison was subjected to a thorough investigation on rabbits by Dreyer and Madsen and gave the following results: If 0.6 cc. poison are mixed with one I. E., it will be found that this mixture, which represents the  $L_0$  dose for guinea-pigs, is still highly toxic for rabbits. In order to render this amount of poison completely innocuous for rabbits it is necessary to add more antitoxin; as a matter of fact it requires  $\frac{240}{200}$  I. E. Their statements concerning the behavior of mixtures between these two limits are also very interesting. A mixture of 0.6 cc. poison +  $\frac{210}{200}$  I. E. given to a rabbit gives rise to paralytic phenomena appearing on the fifteenth day and ending fatally on the twenty-second day. Even a mixture of the same dose of poison with  $\frac{232}{200}$  I. E. produced paralysis commencing on the sixteenth day and continuing for several weeks. In view of the importance of these facts for the conception of a plurality of poisons, I cannot pass on without discussing them more fully. According to our definition of the  $L_0$  dose, such over-neu-

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<sup>1</sup> The explanation of this is that the toxon determination by means of 1 I. E. naturally cannot be an absolutely exact one, small residual amounts of toxin, e.g., 1/10 lethal dose, readily being overlooked. If, however, an appropriate multiple, say ten times this mixture, be injected, this will contain ten times 1/10 fatal dose.

tralized doses, which (like the mixture  $\frac{232}{200}$ ) possess a considerable excess of antitoxin, are absolutely innocuous for guinea-pigs and can be injected in any desired quantity. In fact, owing to the excess of antitoxin, such mixtures furnish the animal with passive immunity and protect it, provided suitable amounts have been injected, against diphtheria poison and diphtheria bacilli. If such mixtures, however, are still toxic for rabbits, only one possibility remains, namely, that the diphtheria poison in question contains a substance which is non-toxic for guinea-pigs, but still toxic for rabbits. This is my toxonoid.<sup>1</sup>

So far as the behavior of partially neutralized mixtures is concerned, the investigations of the two authors show that mixtures which exert only toxon effects on guinea-pigs cause death and symptoms of diphtheria poisoning in rabbits. In my opinion the phenomenon described can best be explained by the assumption that at least three varieties of poison are to be distinguished, possessing different affinities and different actions. Such an assumption, I believe, will best harmonize the actual facts. These poisons are:

1. Toxin, possessing the greatest affinity, kills rabbits and guinea-pigs acutely, but is much more toxic for the former.
2. Toxon, killing rabbits acutely and guinea-pigs with paralytic symptoms.
3. Toxonoids, producing paralyses in rabbits but innocuous for guinea-pigs.

That all these poisons act more powerfully on rabbits than on guinea-pigs is explained by the absolute higher susceptibility of these animals. So far as the behavior of the toxonoids is concerned, in which enormous differences in rabbits and guinea-pigs are manifested, such behavior finds numerous analogies in toxicology, especially in the study of toxins. Thus heroin, an acetyl derivative

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<sup>1</sup> Almost at the outset of my investigations and long prior to Madsen and Dreyer I obtained results entirely similar to these. My unpublished but very extensive studies showed that this property is not possessed by all diphtheria poisons, for I also encountered poisons in which the  $L_0$  dose was exactly the same in guinea-pigs and rabbits. This fact controverts the assumption that perhaps the described phenomenon is due to an incomplete neutralization, such as Arrhenius and Madsen have demonstrated in the union of boric acid and ammonia, and in that of tetanolysin and antilysin. If this were the case one would expect the phenomenon to be present in all diphtheria poisons to the same extent, and this is not the case.

of morphine, is far less toxic for rabbits than is morphine: for asses on the other hand it is far more toxic than the latter substance. In the case of toxins v. Behring long ago showed that for different species of animals certain toxins are very differently affected by trichloriodine. As I suggested in my address at the International Medical Congress in Paris we are evidently dealing here with incomplete toxoids, i.e., with toxoids whose toxophore complex is not yet completely destroyed. Portions of this complex still left to the poison possess a high toxicity for one species of animal and little or no toxicity for another. The toxophore groups of the tetanus poisons mentioned above (Tizzoni and v. Behring) afford a sufficient analogy.

A consideration of these facts will show that Gruber's statement, that the facts observed by Madsen and Dreyer reduce my theory to an absurdity, is absolutely incorrect. On the contrary, I may say that the facts brought out by these authors are most readily explained on the basis of my theory.

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I shall now take up Gruber's recent experiments. These were first published in the *Wiener klin. Wochenschrift*<sup>1</sup> in a form strongly suggestive of the comic supplement of a newspaper.

The discussion takes the form of a letter purporting to be written by a certain "Phantasus," and is really very cleverly conceived. Only I would protest against publications of this sort appearing in the columns of a scientific journal.

Two series of experiments come into question. The first series is so curious that I have not felt any desire to repeat the experiments. These deal (a) with the property of sulphuric acid to act as a poison on cane sugar, and (b) with the antitoxic action which water exerts on this property. Any one with even the faintest knowledge of chemical processes knows that the sulphuric acid as such is not deprived of this poisonous action by water; this is effected only by an alkali which, by forming a salt, neutralizes the acid. I am able to furnish an additional case which shows the "detoxitizing" effect of water. A highly concentrated sulphuric acid, containing considerable anhydride, acts destructively on iron. If  $H_2O$  is added until the solution contains the monohydrate it will be found that the addition of the water has reduced this capacity to attack iron

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<sup>1</sup> *Wiener klin. Wochenschr.*, No. 27, 1903.

to practically zero. In this case then, just as Gruber states, the water has acted as an antitoxin. On the addition of more water to the mixture, however, the iron is again attacked. In fact the more water now added the stronger becomes this action. We thus obtain the curious result that in small doses water acts as antitoxin, while in large doses it increases the action of the poison, surely an interesting problem for Dr. Phantasus!

This is merely one of the special instances of the fact thus far unexplained, that the different hydrates of sulphuric acid, or their mixtures, manifest a most extraordinary variation of properties. I may refer the reader to the minute and fundamental study of Knietseh,<sup>1</sup> in which the variations of the properties of sulphuric acid at different concentrations have been represented in the form of a curve for many of these properties, thus specific heat, electric resistance, boiling point, vapor tension, viscosity, capillarity, action on iron, etc. A glance at this chart gives one the impression of chaos, and at once shows that on these complicated problems only deep studies can lead to any results, and that the ten-minute experiments made by Phantasus-Gruber-Pirquet are absolutely worthless. This is especially true in Gruber's case, which deals with an obscure reaction in which oxidation, abstraction of water, cleavage and sulphurization take part. Hence I deny that crude experiments of this kind can be used to gain an insight into such an entirely different subject, or that the conditions there observed can even be compared to the minutely differentiated processes of toxin-antitoxin combination.

We shall next take up Gruber's experiments which deal with the hæmolytic action of water, since to persons at a distance these might give the impression that they really have something in common with studies in hæmolytic toxins. The experiments are supposed to show that water is composed of an infinite number of different poisons. Let us listen to Gruber for a moment:

"Pure water exercises a very great osmotic pressure on red blood-cells, leading to their swelling and to the escape of hæmoglobin. Hence water is a toxin for the erythrocytes, salt is an antitoxin. When successive amounts of salt are added to the water this toxicity is gradually lost, for the affinity of the water, and with it the osmotic pressure, is thus gradually decreased."

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<sup>1</sup> Bericht d. deutsch. chem. Gesellschaft, 1901, page 4069.



We see therefore that Gruber-Pirquet assume that pure water possesses a high osmotic pressure and that salt diminishes this. The very foundation of the doctrine of osmotic tension, however, is the fact that water as such possesses NO osmotic pressure, and that such pressure is produced by salts dissolved in the water. I cannot refrain from pointing out this woful ignorance of the most elementary principles on the part of authors who do not hesitate to accuse me of "complete lack of insight into chemistry," although for years I have endeavored, and not unsuccessfully, to apply the great discoveries in chemistry to medicine.

The solution of erythrocytes by means of water is one of the best studied subjects in medicine. It is generally recognized that the water as such is no poison whatever, but that its action is due to the fact that water abstracts the salts and other soluble substances from all living cells, including, of course, the red blood-cells. These substances are abstracted in such considerable amounts that this alone suffices to bring about the death of the cell. The swelling of the red blood-cells is due to the penetration of water and this again depends on the permeability of the limiting membrane on the one hand and the power of the water to abstract water on the other.

With the same right that Gruber regards water as a poison one could call nitrogen a poison and oxygen as the counter poison for the nitrogen, for animals die in pure nitrogen, but live if oxygen is added. At any rate nitrogen poison can be recommended to Dr. Phantassus for extended study. Perhaps some day he will also work out its spectrum for us.

Despite the fact that the premises from which their experiment proceeds are based on a complete misconception of the idea of poison, I have repeated the experiments of Gruber and Pirquet. The results show that their statements concerning the experiment are entirely incorrect. I first determined the concentration of salt and of sugar, in which the ox blood-cells remained completely intact; for NaCl this was found to be 0.63%, for cane sugar 6.4%. By diluting with water, various degrees of this isotonicity (1/10, 2/10, etc.) were produced. Each tube contained altogether 2 cc. of fluid and one drop of defibrinated ox blood. The result is shown in the form of a "spectrum," which may be compared to that obtained by Gruber in his experiments.

This comparison shows us that Gruber's experiments are abso-

tely incorrect, and that they contradict all that is thus far known concerning solution of the red blood-cells. Gruber states that in a 1:10 isotonic solution, one containing about 0.07% NaCl, about one-fifth of the blood-cells remain undissolved. All other authors, however, have found that even in a solution of 0.3% NaCl, the blood-cells of all warm-blooded animals are still completely dissolved, so that the solution appears uniformly laky, and microscopical examination shows not even a trace of red-blood corpuscles. In Gruber's spectrum, however, we find that with this percentage more than half of the blood-cells remain undissolved. This indicates that in Gruber's experiments the grossest sort of errors abound.

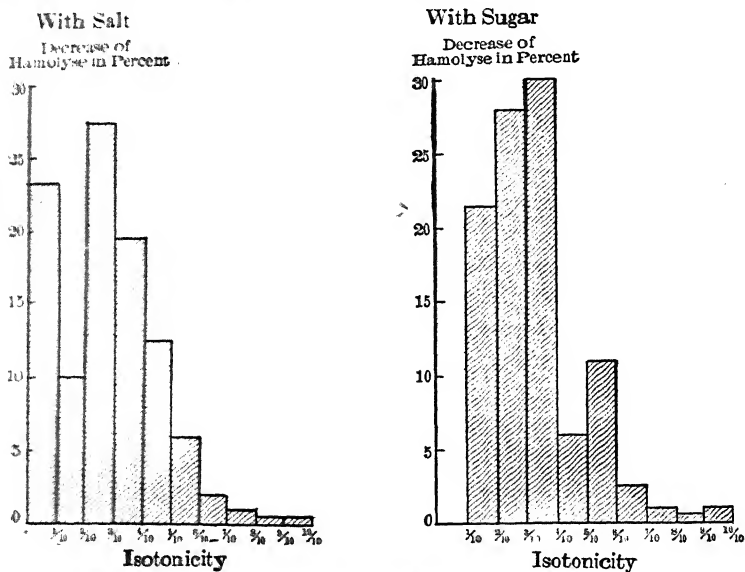


FIG. 1.—“Poison spectrum” of water according to Gruber.

What can we deduce from these spectra? The fact that a certain amount of NaCl can be added to the “poisonous” water without inhibiting hemolysis, would lead authors holding Gruber's views to conclude that this “poisonous” water contains a prototoxoid whose neutralization has no effect whatever on the toxic action. A single glance at the detailed literature on this subject should, however, have convinced these authors that their curve, as such, has nothing whatever to do with toxic actions, but is merely the expression of the specific differences in the red blood-cells. It is well known

that the blood represents a mixture of cells of various ages, and it is not at all surprising, therefore, that these should behave differently toward different injurious influences. We are here dealing with a property of the erythrocyte's protoplasm, which protoplasm will possess a different degree of vulnerability according to its age. Are Gruber-Pirquet entirely unaware that an important and much-employed procedure for determining the resistance of the blood rests on just

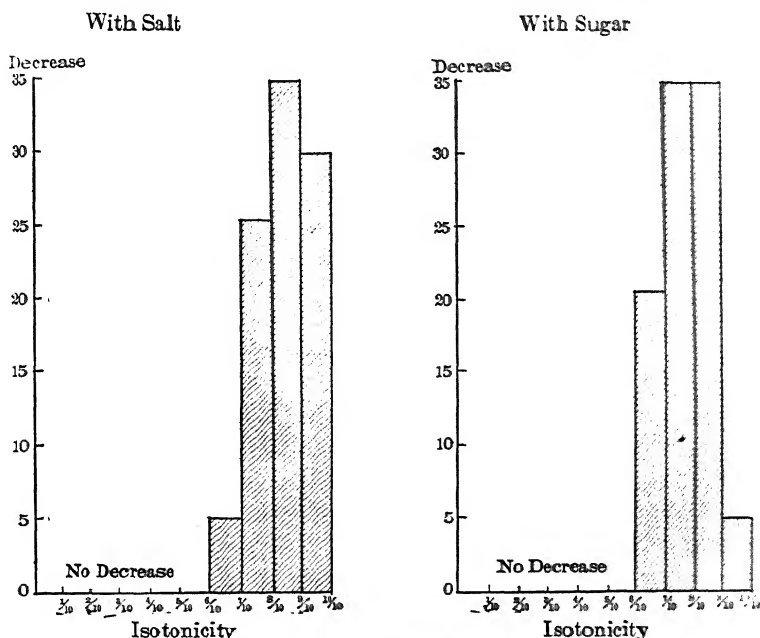


FIG. 2.—"Poison spectrum" of water according to Ehrlich.

this principle? Every text-book on hæmatology teaches that we distinguish blood-cells of maximum, minimum, and intermediate resistance, and that the extent of resistance is merely the difference between the maximum and minimum.

Instead of this, however, Gruber feels compelled to draw from his curves conclusions having such far-reaching consequences as, for example, that water is full of poisons, of haptophore and toxophore groups, etc. But if he believes that this proves the folly of my conception of toxin neutralization, so much the worse for him and his authority Phantassus.

If one conducts experiments that have nothing to do with the problem under discussion, further, if the method of these experiments is grossly at fault, and if, finally, the results thus obtained are given an utterly false interpretation, it is not surprising that the most fantastic results are obtained.

Finally Gruber describes one more experiment which he illustrates by means of a curve. According to him this too demonstrates that my theory is untenable. The experiment shows that the hæmolysis of ox blood, by means of a certain quantity of specific hæmolytic serum within half an hour, is dependent on the dilution. I need hardly remind my readers that I have always laid stress on the chemical nature of the toxin and antitoxin combination. I can assure them that the factor of the degree of concentration has ever been sufficiently regarded. If Gruber will refer to my first study on this subject, "*Die Werthbemessung des Diphtherieheilsersums*," he will find the statement: "that the union of poison and antibody proceeds much more rapidly in concentrated than in dilute solutions," and further also "that heat hastens the union and cold retards the same."

The behavior which Gruber describes is all the less surprising since he is dealing with a complex process depending on the action of the amboceptor-complement combination. How readily this combination is dissociated has repeatedly been pointed out by us. Perhaps Gruber thinks that this experiment is new to me; every one versed in the subject, however, knows that we are here dealing with the most commonplace phenomena, with which every beginner is well acquainted. I should like to point out, however, that this phenomenon, namely, that dilution with water inhibits the action of hæmolysins, is not at all constant. On the contrary it is limited to those cases in which the affinity between amboceptor and cell, or between amboceptor and complement is relatively slight. If one employs poisons in which the affinity between receptor and cell is great it will be found that within the limits mentioned the addition of water is practically without effect. Thus, in working with cobra venom, I found that a given quantity of this poison exerted exactly the same effect whether the volume of water used was 1 or 15.

It would lead us too far to enter into all the distortions and misconceptions contained in Gruber's polemic. To do this would require almost a complete reprint of all my articles, as well as of many others

emanating from the Institute—with all of which Gruber seems quite unfamiliar. I shall content myself therefore with a brief discussion of Gruber's conclusions. Gruber states:

1. "There is no warrant for assuming that the bacterial toxic solutions contain a number of poisons possessing qualitatively similar actions but differing in intensity and in their affinity to the antitoxin."

In the preceding pages I have conclusively shown that his view cannot be harmonized with the actual facts. But even *a priori* there is no reason to assume that bacterial cells always produce only a single poisonous metabolic product. Thus, to mention only a few examples, we know that cinchona bark contains about twenty different alkaloids, opium about the same number; Flexner and Noguchi's researches show that snake venom contains at least four different poisons (hæmotoxin, leucotoxin, neurotoxin, endotheliotoxin), and the yeast cell, we know, contains a number of different ferments. Furthermore, I may again call attention to the fact that the secretion of tetanus bacilli contains four distinct poisons, namely, two varieties of tetanospasmin, my tetanolsin, and the poison which, according to Tizzoni, causes the cachexia. So far as diphtheria poison is concerned the reader is referred to my previous statements. My assumption of the existence of at least two poisons, toxins, and toxons, is borne out by the clinical observation that in certain epidemics there is a large percentage of paralyses.<sup>1</sup>

2. "There is no reason for assuming that the mode of action of the toxins is absolutely unlike that of other organic poisons."

Nevertheless, the fact remains that the principal characteristic of the toxins, namely, the production of antibodies, does differentiate them from all other poisons, Gruber to the contrary notwithstanding. Two years ago Gruber could have found an ally in Pohl, who

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<sup>1</sup> In animal experiments as a rule, the toxons do not manifest themselves until the toxins (which possess a greater affinity) have been neutralized by the antitoxin. Dreyer and Madsen, however, have described a diphtheria poison (Festskrift, Copenhagen, 1902), in which the toxons could be demonstrated even by the injection of sublethal doses, the injections being followed by paralytic phenomena. In view of the constants of this poison, as they were determined by Dreyer and Madsen, this behavior is not at all surprising, for while old diphtheria bouillons ordinarily contain about 33 toxon equivalents to 167 toxin equivalents, this poison contained about 500 toxon equivalents for that amount of toxin.

had apparently succeeded in immunizing against solanin. Since then, however, the researches of Bashford<sup>1</sup> and of Besredka<sup>2</sup> have shown that it is impossible to produce antibodies against either solanin or saponin. Pohl himself no longer maintains the existence of a specific antisolanin. Of the various poisons, which seemed to promise the best for successful immunization, morphine should be mentioned first. Recently Hirschlaff<sup>3</sup> claimed actually to have produced an antimorphine serum. Morgenroth,<sup>4</sup> however, was able to show that the results obtained by Hirschlaff were merely apparent, not real, and that they depended on the fact that the doses of poison employed by Hirschlaff were not surely fatal, especially owing to the increased resistance of the animal following the serum injection. Hence the statement still holds true that all poisons chemically well defined do not possess the property of producing antitoxins.

So far as other differences between ordinary poisons and toxins are concerned, I may refer particularly to my detailed articles in von Leydens Festschrift<sup>5</sup> and to the excellent monograph by Overton.<sup>6</sup> From these it will be seen that the action of the chemically defined poisons, alkaloids, glucosides, etc., on parenchyma is the result of a solid solution or of a loose salt formation. In accordance with the loose character of the combination, the action of these poisons is a transitory one. The firm union and prolonged action peculiar to the toxins is entirely absent. Besides this the period of incubation is wanting in most ordinary poisons, although there are a few exceptions like arsenic, phosphorus, tartrate of tin and sodium, and vinylamin. In the toxins, on the other hand, a period of incubation is the rule.

Entirely in accordance with the views of Emil Fischer concerning ferments, I have ascribed the specific combining processes of toxins to certain stereochemical groups of atoms (haptophore groups). These unite only with such other atomic groups which fit to them as does a key to a lock. The ordinary chemical groups of organic chemistry possess affinities for a large number of other groups. Thus

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<sup>1</sup> Archives Internationales de Pharmacodynamies, Vols. 8 and 9.

<sup>2</sup> See Metchnikoff, L'Immunité, Paris, 1901.

<sup>3</sup> Berliner klin. Wochenschrift 1902.

<sup>4</sup> Ibid., 1903, No. 21.

<sup>5</sup> Von Leydens Festschrift, August Hirschwald, Berlin, 1902.

<sup>6</sup> Studien über die Narkose, Jena 1901.

the aldehyde group can unite with amido groups, hydrazin groups, methylen groups, etc. In this group therefore the combining property is not specifically limited, but extends to a large number of combinations. On the other hand the one characteristic of toxins and ferments is just this *specific* combining property.

3. "The transformation of toxins into non-poisonous combinations (toxoids), possessing the same affinity for the antitoxin is possible, but has not been definitely proven."

I have already clearly shown that the doctrine of toxoids, now generally accepted, is one of the best-established foundations in the entire subject of immunity. However, with critics like Gruber, who blindly condemn the views of others, one ought to be satisfied if they recognize at least a possibility.

4. "Toxin and antitoxin have feeble chemical affinities and therefore unite with one another to form dissociable combinations or perhaps molecular combinations in varying proportions. These conditions explain the long incubation of the poisonous action and other marked phenomena."

To be sure the affinity between toxin and antitoxin may in some instances be a feeble one, but this is by no means always the case. The affinity between tetanus toxin and antitoxin is slight, and so is that between complement and amboceptor. On the other hand, however, there are poisons, such as diphtheria toxin and snake venom, in which the reaction proceeds under strong affinities, so that the process of neutralization takes the course of a straight line and not of a curve.

Gruber's statements might also give one the impression that he is the first to introduce dissociation as an explanation of some of the phenomena in immunity. I have always emphasized the fact that amboceptor and complement are loosely bound, uniting at high temperatures, but dissociating at low temperatures.<sup>1</sup> But this is all wrong according to Gruber,<sup>1</sup> for a year and a half ago he

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<sup>1</sup>I shall cite a passage from Ehrlich and Morgenroth's First Communication Concerning Hæmolysins (see page 7 of this volume), a passage which Wechsberg has already called to Gruber's attention (Wiener klin. Wochenschr. 1901, No. 51). "This experiment clearly shows that under the conditions present complement and immune body exist in the serum independently of one another"; further also, "under certain circumstances the immune body enters into a loose chemical union with the complement, one which is easily dissociated." In view of this I cannot understand why Gruber still main-

laid down the dictum, "There is no dissociation by means of cold." It seems not to have mattered to him that his statement is opposed to even the most elementary principles of chemistry.

As a matter of fact we have always paid due attention to dissociation and to the reversibility of the reactions. I should like to call Gruber's attention to the fact that the sentence: "In the union of the amboceptors we are dealing with a reversible process" occurs in one of Morgenroth's studies<sup>2</sup> from this Institute. Further than this such questions do not affect the Side-chain Theory, as such. The whole discussion is evidently designed to hide the fact that Gruber's position is really based on my theory.

So far as the mode of action of the toxins is concerned, Gruber's standpoint and mine are essentially the same. Thus Gruber states that: "All poisons must be 'anchored' by the cells and the anchoring group of atoms is probably always different from that group which gives the substance its toxicity." I spent many years in establishing this view and it is now everywhere accepted as axiomatic. I defy Gruber to show me the text-books of toxicology in which, previous to my work, this conception appears, a conception which dominates the laws of the distribution and action of poisons. If he should again refer to S. Fränkel's book<sup>3</sup> I can only remark that while the account of my views is very admirable, it is nothing more than a résumé of the points which I had previously developed. Perhaps I can even aid Gruber's memory and let him speak for himself. A year before his declaration of war he spoke of "the brilliant hypothesis of that genius Paul Ehrlich, the greatest of living pathologists." In a little work<sup>4</sup> published at that time, and quite enthusiastic over my theory he states: "According to Ehrlich only such substances are poisons which unite chemically with some constituent of the organism." And yet this same Gruber to-day says: "These are merely new words for what has long been known."

I should not like to deprive the reader of hearing still another

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tains that my view of the production of anticomplements, according to which amboceptor and complement are *firmly* united, is absolutely incomprehensible.

<sup>1</sup> Münch. med. Wochenschr. 1901, No. 48.

<sup>2</sup> Ibid., 1903.

<sup>3</sup> Die Arzneimittelsynthese, Berlin, 1901.

<sup>4</sup> Max Gruber. Neuere Forschungen über erworbene Immunität, Vienna, 1900.



authority often cited by Gruber, namely von Behring. Shortly after my theory was formulated this author expressed himself as follows:<sup>1</sup> "It seemed about hopeless to attempt to penetrate these mysteries, when recently Prof. Ehrlich published a theory which is destined to illuminate even this subject."

But even now Gruber does not doubt "that the toxins are very complex bodies and that the toxic action is connected with certain atomic groups; that possibly it is necessary for certain atomic groups to be present so that the poison molecule can be anchored and the toxicity manifest itself."

One will at once ask why then Gruber attacks my theory if he is satisfied with its fundamental principle, namely, the assumption of an independent haptophore and toxophore group in the poison molecule? That I cannot answer. To be sure further along one encounters the warning, "But one must not too highly personify these different atomic groups, and think of this entire poisoning as a drama with four long intermissions between the acts." I cannot see what is to be gained by such idle talk.

As a matter of fact the majority of infectious diseases as well as the poisonings do proceed in three phases, and these have always been separated, namely, incubation, the disease itself, recovery. Hence to explain these, as we do, through the independent action of toxophore and haptophore groups seems the most natural thing to do. It is strange that Gruber should now speak of the anchoring of the poison by the elements susceptible thereto as something perfectly obvious, for in his first attack he laid especial emphasis on "his being the first to furnish the important demonstration that the specific immune substances are bound by the bacteria." However, Gruber's claim cannot be allowed, for all that he demonstrated was that the agglutinins are used up in the reaction. The significance of a chemical union, however, was first pointed out by us. This union, as Morgenroth's studies on the behavior of anchored amboceptors show, need in no way be connected with toxic action or with a using up of the substance.

Gruber's statement that the long period of incubation is explained by the feeble affinities I must emphatically deny. The studies of Dönitz<sup>2</sup> and of the Heyman school<sup>3</sup> show that the injected toxins

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<sup>1</sup> Deutsche med. Wochenschr. 1898.

<sup>2</sup> Ibid., 1897.

<sup>3</sup> Decroly et Rouse, Arch. de Internat. de Pharmacodynamie, Vol. VI.

disappear from the circulation in a few minutes. It is therefore idle to talk of a slow union such as would correspond to weak affinities. But, says Gruber, "it is impossible to understand why the toxophore groups, after they have been brought into proximity to the protoplasm, do not at once commence their activity, but always stop to consider the matter for several hours." One cannot seriously discuss the subject with such a questioner. Gruber might just as well ask that all chemical reactions proceed rapidly, and deny the possibility of a slow reaction.

The slow action of the toxophore group is not at all remarkable, especially in the domain of toxins. This is particularly true if we remember that with certain poisons (e.g. botulism toxin), one part of toxin to 500 million parts of body weight suffices to cause death, and that the rapidity of action is dependent to a high degree on the amount of the active substance.

Is Gruber possibly of the opinion that in the paralysis of diphtheria, which as is well known usually develops after the lapse of weeks, the toxon courses about free for twenty days or more before entering the tissues and then suddenly exerts its action? To the unprejudiced critic the importance of the separation of toxin binding and toxin action for the proper understanding of the period of incubation, is conclusively demonstrated by Morgenroth's<sup>1</sup> experiments with tetanus in frogs. Courmont and Doyon, as is well known, discovered that the frog is susceptible to tetanus poison only at higher temperatures, and not when the animal is kept cold. Morgenroth was able to show that at low temperatures the tetanus poison is bound, but exerts no toxic action. Frogs are injected with tetanus toxin and then kept on ice for days. If then they are subjected to higher temperatures, it will be found that they behave exactly as if they had just been inoculated. And yet the toxin has been bound by the central nervous system even at the low temperature; for if after several days at low temperature the animal be injected with an amount of antitoxin, even much more than sufficient to neutralize the poison, tetanus will still develop if the frog is subjected to a higher temperature. But this is not all. If frogs, after being injected with tetanus, are subjected to a high temperature for one day, and then placed in the refrigerator, they will not become sick. But on bringing the animals back into higher temperatures after

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<sup>1</sup> Arch. Internat. de Pharmacodynam., Vol. 7, 1900.

the lapse of weeks or months, it will be found that they sicken after a shortened period of incubation. Are any further proofs of the slow action of the toxophore group required?

It is not easy to meet all of Gruber's statements because he frequently makes use of misleading tactics. He often reaches the same conclusions as I myself, and grants that certain of my views are permissible or probable. In some things, he says, I am correct in the main, in others I may be right, but have not strictly proved my point. All these statements are but a clever contrivance to give the reader the impression that my theory is but a product of the imagination when as a matter of fact is it really a hypothesis developed experimentally. This brings me to Gruber's fifth conclusion.

5. "The development of antitoxin has no connection whatever with toxic action or cell immunity."

It will suffice for me to call attention to the fact that I have always insisted on distinguishing between the haptophore and toxophore groups in the toxin molecule and also between the anchoring and the action of poison. I might add that this absolute independence of toxic action and antibody production is a principle which I formulated, not Gruber. As far back as 1898, Weigert<sup>1</sup> rightly pointed out that my demonstration<sup>2</sup> of antitoxin production through non-poisonous toxoids was sufficient to demonstrate the independence of antitoxin production and toxic action. Furthermore I have repeatedly pointed out that the development of antitoxin depends on the haptophore group. Over 1½ years ago Paltauf<sup>3</sup> called Gruber's attention to the weak points in his objection and one might therefore have expected that Gruber would not again bring forward this old fairy-tale. In the future I shall not reply to perversions of this kind.

So far as the reasons are concerned, which Gruber gives in support of the above statement regarding the development of antitoxin, I may at once say that I can assent to them word for word. Thus the statement that:

(a) "Many substances which are entirely innocuous lead to the formation of antibodies" is the first consequence of my views and experimental labors. The fact that

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<sup>1</sup> Lubarsch-Ostertag, *Ergebnisse der pathologischen Anatomie*, IV Jahrgang.

<sup>2</sup> *Werthbemessung des Diphtherieheilserums*, *Klin. Jahrbuch*.

<sup>3</sup> *Wiener klin. Wochenschr.* No. 49, 1901.

(b) "Certain animals non-susceptible to certain toxins nevertheless produce antibodies" needs no further explanation according to my theory. Certain species of animals may possess suitable receptors for binding the toxin and producing antitoxin although their cells are insensitive to the action of the toxophore group. According to Metchnikoff this seems often to be the case with tetanus toxin in crocodiles. As already pointed out years ago by Weigert<sup>1</sup> according to my theory, the production of antitoxin need not at all be preceded by any injury in a clinical sense. In fact, too strong an injury may cause the cell to lose its power of regeneration, owing to the toxic action on the vital group [Leistungskern]. For example, if a specific nerve poison is anchored by a fitting receptor of an indifferent cell (liver) we should expect the production of an antibody by the liver, even if the liver-cell does not become tetanized. In my address at Hamburg<sup>2</sup> before the Congress of Naturalists I pointed out that the local origin of antitoxin, which Römer deduces from his splendid experiments with abrin, will often make it possible to transfer part of the antitoxin production from the vital organs to the indifferent connective tissue, by means of subcutaneous injection of poison.

Gruber's next statement is:

(c) "Despite a plentiful production of antibody, the susceptibility to the poison may remain, or even increase."

I have already discussed the principle of hypersensitiveness and mentioned the fact that this objection restrained me for a long time from publishing my theory. But even these phenomena were satisfactorily explained in accordance with the side-chain theory, by the assumption of an increase of affinity and a rupture of the toxin-antitoxin combination. To be sure it is possible that our explanation touches but part of the subject, and that in reality the phenomena are far more complex. But this is no reason for seeking to overthrow the theory; to do so would be to completely misapprehend the purpose of a theory. Surely one cannot demand that a theory will at once explain all the complex phenomena of so difficult a subject as this. A theory ought primarily to possess heuristic value, pointing out new paths into a complex subject; it should smooth the way. The actual research must be left to the scientific investigator. Science can be advanced only by means

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<sup>1</sup>l. c.

<sup>2</sup>Deutsche med. Wochenschr. 1901.

of experimental analysis, and not by high-flown words of a misleading dialectic.

(d) "Cell immunity can be acquired without the formation of antibodies."

This statement, too, does not surprise me. All that the side-chain theory aims to do is to explain how the production of antibodies may be conceived. But I have never yet claimed that this is the only means by which the organism can defend itself against deleterious influences. I would call attention particularly to the Sixth Communication on Hæmolysins,<sup>1</sup> in which Morgenroth and I pointed out that not all substances capable of being anchored need necessarily excite the production of antibodies. We have always emphasized, however, that immunity may be developed despite this, chiefly through a disappearance of receptors.<sup>2</sup> In our isolysin experiments we observed that the blood-cells became insusceptible and we demonstrated that this was due to a lack of receptors. The interesting fact observed by Kossel and by Camus and Gley that during the course of immunization with eel blood, the blood-cells of rabbits acquire a high resistance against that poison, is probably most easily explained by assuming that the cells acquired immunity in the way above mentioned.

This, of course, does not exhaust the possibilities of the origin of immunity not due to antitoxins. Thus under the influence of the anchored poison new receptors may be formed which are so firmly united to the protoplasm that they are not thrust off. Such receptors Morgenroth and I have therefore termed "sessile receptors." If the production of such an excess of receptors takes place in a rather indifferent tissue, as in connective tissue, it will readily be seen how the receptors can serve to deflect the poison, and produce a more or less marked immunity. In that case on comparing a normal animal with an immunized one, the conditions would be like those observed with tetanus poison in normal guinea-pigs and normal rabbits, respectively. The studies of Dönitz and Roux have shown that the guinea-pig possesses receptors for tetanus toxin only in the brain, whereas, rabbits, in addition to the receptors in the central nervous system, possess about thirty times as many such receptors outside this system.

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<sup>1</sup> See page 88.

<sup>2</sup> Schlussbetrachtungen in Nothnagel's Handbuch., Vol. VIII.

Another possibility of cell immunity is that the protoplasm of cells which are ordinarily susceptible is no longer affected by certain poisons. This kind of immunity, which to be sure I consider very rare, would correspond to mithridatism or acquired tolerance in the old sense. A fourth possibility, finally, is the adaptation of the phagocytic apparatus in Metchnikoff's sense.

It is obvious, of course, that all the sevarious subordinate kinds of immunity occur alone as well as in manifold combinations. Thus, as already mentioned, immunization with eel blood is followed by antitoxin immunity and tissue immunity. In the lower animals, however, which as Metchnikoff has shown are but little adapted to the production of antitoxin, other defensive contrivances leading to cell immunity will predominate. From this point of view therefore the condition described by Gruber, namely, that frogs can be immunized against abrin without their showing any antitoxin, offers no difficulty. So far as the frog is concerned the only question is which kind of cell immunity is present, i.e., whether there is a disappearance of receptors, or whether there are sessile receptors, etc.<sup>1</sup>

In view of the detailed statements given above I presume I need add nothing to the following passage in Gruber's conclusion:

(e) "The production of antibodies takes place at entirely different localities than does toxic action."

The discerning reader will at once see that this statement does not in the least contradict my views. In fact it is merely another way of expressing what is really the nucleus of my theory. The generalization, however, is false, that the production of antibody necessarily takes place in localities different from those in which toxic action occurs. If Gruber therefore believes that this riddles my theory it is evident that he understands the principles under-

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<sup>1</sup> Gruber cites, as a serious objection to my theory, that Madsen observed immunity in a rabbit which had been immunized with diphtheria toxin, and yet was unable to find antitoxin in the blood. I will only say that Madsen did not find the blood entirely free from antitoxin since he tested the serum only to 1/10 I. E. Small quantities of antitoxin could be very well have been present and these, of course, would be of considerable importance for the question as to whether this was a case of entire absence of antitoxin. Besides this I may add that in diphtheria poison the case reported by Madsen must be extremely rare. During the course of many years the different Serum Institutes have immunized thousands of different animals against diphtheria. In all this time, however, I have never learned of a case analogous to Madsen's, either from the literature or from private sources.

lying my views no better than he did two years ago. At that time Paltauf<sup>1</sup> tried in vain to make this elementary consequence of the side-chain theory comprehensible to him.

Gruber's sixth conclusion is as follows:

6. "The specific antibodies are not normal body constituents. They are newly formed only after the introduction of foreign substances. This new formation has the character of an internal secretion."

So far as the first point is concerned one cannot help being amazed at the lack of literary knowledge which permits an author to make such statements. I need only refer to the studies of Pfeiffer, Bordet, Flexner, Kraus, Bail, Peterssen, etc., or to the comprehensive résumé by M. Neisser<sup>2</sup> concerning the antibodies found in normal serum. The literature on normal antibodies of various kinds is very large, and yet has been entirely ignored by Gruber. Thus amboceptors against different bacteria (cholera, typhoid, anthrax), antiamboceptors, anticomplements, antitoxins, antiferments, etc., have been observed. I shall, however, mention merely a few points which may be of special interest.

I. The very frequent occurrence of diphtheria antitoxin in horses (Meade, Roux, Bolton, Cobbett). In view of the high percentage of this occurrence, the attempts to ascribe this antitoxin in normal horse serum to a diphtheria running a latent course must be regarded as failures. Since this phenomenon has been observed in about 30% of the horses, it is surely not reasonable to assume that an occurrence of diphtheria in horses should so frequently have entirely escaped the large number of excellent observers representing animal pathology. Such a frequency of the disease should, of course, also have manifested itself epidemiologically. The fact that in one single instance Cobbett observed a diphtheritic infection in a horse certainly does not alter the circumstances.

II. I must mention the interesting observations made by v. Dungere<sup>3</sup> that normal rabbit serum contains an antibody against that substance in star-fish eggs which is toxic for sea-urchin spermatozoa. I am sure that no one, just to please Gruber, will assume that there is any connection between rabbits and star-fish and their eggs.

III. Laveran has found that the blood of healthy human beings

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<sup>1</sup> Wiener klin. Wochenschr. 1901, No. 49.

<sup>2</sup> Deutsche med. Wochenschr. 1900.

<sup>3</sup> Zeitschr. f. allgemeine Physiologie, Vol. 1, 1901.

contains a substance which kills trypanosomes, whereas this is not present in the blood of other animals and cannot be produced in so large an amount even by immunization. This might be the reason why (aside from sleeping sickness of Central Africa) man is so refractory toward trypanosome infection.

But if such a wealth of facts is disregarded in statements concerning "our certain knowledge," it must be admitted that a scientific discussion is entirely out of the question, and had best be avoided in the future.

Furthermore, so far as conceiving the production of antitoxin to be a secretion is concerned, I may say that this part of the paper is nothing but another way of stating what I have always held. Paltauf,<sup>1</sup> for instance, pointed this out to Gruber some years ago, "In passing I may say that an 'escape' of particles of protoplasm into the blood really denotes a secretion." In an address delivered in 1899 (!) I expressed myself in a way that shows that I have always considered the production of antitoxin to be a secretory process.<sup>2</sup>

"Or, s'il y a lieu de croire que les Antitoxines doivent leur origine à une sorte de fonction sécrétoire des cellules et ne sont par conséquent nullement étrangères à l'organisme, le rapport spécifique qui les unit avec leurs toxines n'en devient que plus étrange."

This point has been demonstrated especially by the researches of Salomonsen and Madsen, and of Roux and Vaillard.

But just this secretory character of antibody production is absolutely at variance with the older view that antitoxins are merely transformation products of the toxins. This was the view defended by Buchner and held to be possible by Gruber even in his last attack. It is just as impossible to believe that antitoxins arise from toxins as it is to believe that lipase is transformed fat, or amylase, transformed starch.

Thus we see that the various points brought up by Gruber are nothing but reproductions of my views; the little that deviates is incorrect or is based on misconceptions of an inflated knowledge of the literature.

Gruber's last two conclusions contain so little that is new that it hardly pays to discuss them. For completeness' sake, however, I shall append them.

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<sup>1</sup> Wiener klin. Wochenschr. 1901, No. 49.

<sup>2</sup> This appeared only as an abstract in *La Semaine Médicale*, 1899.



7. "The power to excite the formation of antibodies is due to certain peculiarities in the chemical structure of the substance which excites this antibody production. A prerequisite for this production as well as for toxic action is the chemical union of the foreign substance with certain particular constituents of the cells."

This, I may say, is a short, though not particularly good, résumé of the side-chain theory.

8. "The non-poisonous toxin-antitoxin combination also lacks the power to excite the production of antitoxin. The entire chemical character of this combination is different from that of the uncombined substances."

This, too, is one of the fundamental principles of my theory, and is most readily explained by the assumption that the antitoxin fits into the same group which effects the union of the toxin with the susceptible cells. Furthermore, I really see no reason why Gruber should make a special point of the fact that the chemical character of the toxin-antitoxin combination has changed. That is merely a trick of speech which will make but little impression on the scientific reader.

That the antitoxins are nothing but thrust-off receptors capable of uniting with the poison—this assumption, together with its immediate consequence that the toxin-antitoxin combination must be non-poisonous, is the key to my entire theory. We are, in fact, dealing with an extremely important law which Weigert and I compared to the principle of the lightning-rod and which v. Behring briefly expressed as follows: "The same substance in the living body which, when in the cell, is the prerequisite of a poisoning, becomes the healing agent when it is present in the blood." This law applies not only to the toxins but possesses general applicability. I may here refer the reader to Ransom's experiments, which show that the cholesterin in the red blood-cells causes hæmolysis by saponin, while at the same time the cholesterin of the serum causes an inhibition of this poisoning.

Gruber, however, thinks that it has not been proved that the haptophore group, which anchors the toxin to the vital constituent of the protoplasm, is the same which anchors the toxin to the antitoxin. A year and a half ago he expressed this quite clearly as follows:<sup>1</sup>

"Ehrlich may have demonstrated that the toxin is bound to

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<sup>1</sup> Wiener klin. Wochenschrift, 1901, No. 50.

the antitoxin by a combining group which differs from the toxophore group. But where and how has he shown that the toxin in addition to its toxophore group possesses only *one* haptophore group, namely, the one which combines with the antitoxin? How has he shown that the *same* haptophore group acts in all chemical reactions of the toxin? On the contrary it can positively be stated that the toxin must necessarily be a very complex molecule possessing *many* different haptophore groups. Here, gentlemen, lies the root of the evil. All this misconception of the side-chain theory would have been impossible but for the mistake in the choice of an article; i.e., if Ehrlich instead of speaking of *the* haptophore group had said *a* haptophore group."

So this is my great fault, the choice of an article! I may leave it for the reader to decide how weighty this objection is. Nevertheless let us see what Gruber really means.

Let us assume, for example, that a poison, in addition to the toxophore group, possesses two different groups with haptophore functions. One of these, group *a*, corresponds to what my theory demands, since it is able to combine with a receptor of the cell. As a result of this combination, however, there is to be not an overproduction of a receptor fitted to *a*, but the production of a different substance, fitting the second haptophore group, *b*, of the toxin. It will at once be seen that this entire premise of Gruber is very artificial and unnatural. One can easily understand that the blocking of a given group can cause a new development of the same group. This corresponds to Weigert's fundamental law of regeneration. But it is very difficult to comprehend how the blocking of one group, *a*, would always lead to the development of a different group, *b*. Furthermore, it is incomprehensible why at least part of the poison by means of its haptophore group *b* should not be anchored by a combining substance preformed in the cell, a substance which can therefore act as a receptor. If the toxin really possessed two haptophore groups, *a* and *b*, it would be possible and probable that two different antitoxins would be developed by the cell. But that is a question easily decided experimentally, and one which has been studied in this Institute for years. During all this time we have never discovered even the slightest reason for believing that diphtheria serum, obtained from different animals and by means of different cultures, possesses any such complex constitution as Gruber's view would require.

We see, therefore, that the first step taken with the aid of Gruber's hypothesis leads us astray. But when we attempt to see how the antitoxin could act according to Gruber's scheme, we find ourselves lost in a maze. The antibody secreted by the cell is to combine with a collateral group *b*, of the toxin, leaving group *a*, which primarily effected the anchoring of the poison, intact. How then is any antitoxic effect to take place? One might perhaps assume that by the occupation of group *b*, the toxin loses its toxicity through some influence exerted on the toxophore group. The poison would thus in a certain sense be changed into a toxoid by the occupation of group *b*. In that case, however, the toxin with group *b* neutralized, should still be able to excite the production of antitoxin, just as toxoids do. As a matter of fact, this is not at all the case, for we know that toxin neutralized with antitoxin has completely lost both its toxic property and its power to produce antitoxin. This fact, which is absolutely irreconcilable with a plurality of the haptophore groups, is easily explained by my theory by a blocking of the haptophore group of the toxin.

We see, therefore, that Gruber's assumption leads to consequences which are absolutely untenable. It certainly is far from being an improvement on my theory. In general, also, the principles of scientific investigation demand that we restrict ourselves to the simplest explanations possible and only make use of more complex ones if it is absolutely necessary. But there is not the least reason for Gruber's assumption of several haptophore groups; on the contrary there are a large number of objections to it.

By this I do not mean to say that in addition to the haptophore and toxophore group the toxin molecule contains no other chemical groups, such as amido or aldehyde groups, which are able to combine with other bodies. I merely contend that these atomic groups do not influence the specific immunizing process.

To take a chemical example, it is possible by diazotizing all kinds of amines to transform these into diazo combinations which, corresponding to the original substance employed, contain other radicals capable of reacting, thus COH, CN, OH, NO, etc. The specific property of these substances, that is, the property of forming azo dyes, is, however, connected exclusively with the N-N group. The reactions which the other groups can enter into have nothing to do with this specific reaction. I conceive the constitution of the toxins to be similar in character.

A few words, now, concerning the side-chain theory and immunity. Gruber himself has found that this theory is constantly gaining ground, while I am gratified to see it treated in detail in the best text-books as well as in excellent digests compiled by a large number of my colleagues.<sup>1</sup> In addition to this hundreds of separate studies have been based on the side-chain theory so that I may well believe that it best serves to explain the facts already observed as well as to allow new facts to be predicted. Gruber's appeal,<sup>2</sup> therefore, that "Ehrlich's theory is a great mistake, and is bound soon to disappear from the scientific arena," has had but little success; in fact it seems to have had the contrary effect. The large number of investigators, who are constantly eagerly working on the problems of immunity know what is best for them, and will not be dictated to against their own experience and conviction by one who seeks to make up his own lack of experimental work in this complex domain, by superficial studies of the literature. Gruber, for instance, says that his original failure was due perhaps to the fact "that a few of his experiments proved not to be quite sufficient." This is a mild expression in view of the fact that every one of Gruber's experiments directed against my views has been shown to be fallacious. The studies in which his errors were pointed out and demonstrated experimentally have all been published in detail.<sup>3</sup> The result, as usual, was, that after the corrections had been made, Gruber's attacks proved to be additional supports for my theory. Gruber has not replied to these articles, despite the long time since their publication. Perhaps he thinks the less said the better.

I have finished. I must almost wonder why this detailed reply to an attack whose virulence and unusual tone are almost a confirmation of my views. But I have thought it my duty to guide the reader through the intricate maze of Gruber's statements because I feel that, owing to the large number of misconceptions and misleading arguments which they contain, a field of investigation full of promise might become discredited.

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<sup>1</sup> I may mention those of Aschoff, v. Dungern, Grünbaum, Levaditi, Sachs Tavel, Wassermann, Welch, Bruck.

<sup>2</sup> Wiener klin. Wochensch. 1901, No. 44.

<sup>3</sup> Sachs, Berl. klin. Wochensch. 1902, Nos. 9 and 10; Ehrlich und Sachs, same journal, 1902, No. 21; Morgenroth and Sachs, same journal, 1902, Nos. 27 and 35; Marx, Zeitsch. f. Hyg., Bd. 40, 1902; Wechsberg, Wiener klin. Wochensch. 1902, Nos. 13 and 28.

## XXXIX. THE RELATIONS EXISTING BETWEEN TOXIN AND ANTITOXIN AND THE METHODS OF THEIR STUDY.<sup>1</sup>

BY

Prof. PAUL EHRLICH and DR. HANS SACHS.

THE subject of toxins and antitoxins, although representing one of the best studied domains of biology, is still the subject of lively controversy. The difficulties which beset exact studies are obvious. We are dealing with substances which, for the present at least, are of unknown chemical constitution and which we are compelled to employ in the form presented by the life activities of vegetable or animal organisms, i.e., in an impure state and mixed with countless other products of the living body. All attempts to isolate these bodies and discover their chemical character encounter endless difficulties, so that, if we consider their great significance in practical medicine, it almost seems ironical for nature to offer these substances to man in such an unstable and variable form. In spite of this, however, scientific investigations have been able to obtain a deep insight into the nature and mode of action of toxins and antitoxins; and since chemical means could not be employed, it remained for the experimental biologist to undertake these studies. In place of chemical analysis, therefore, we have the biological reaction, which in the case of toxins is the characteristic toxic action, in the case of antitoxins the property of specifically influencing or inhibiting this action.

An event of considerable importance was the introduction of the quantitative method of study by Ehrlich, a method which opened the way for the present development of immunity studies. At the same time Ehrlich's introduction of test-tube experiments (hæmagglutination, hæmolysis), by avoiding the individual fluctuations of

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<sup>1</sup> Über die Beziehungen zwischen Toxin und Antitoxin und die Wege ihrer Erforschung. Leipzig, 1905, Gustav Fock.

animal experiments, furnished a more exact basis, so that the mathematical harmony of toxin-antitoxin experiments *in vivo* and *in vitro* became very convincing. At the present time, therefore, we may regard it as almost axiomatic that toxin and antitoxin act on each other chemically and without the intervention of vital forces.

These quantitative biological studies, however, have not merely thrown light on the relations existing between toxin and antitoxin, but have also given us valuable information concerning the constitution of the poisons themselves. Almost at the outset it was found that the two properties of toxins which could be analyzed, namely, poisonous action and the property to bind antitoxin, do not at all go hand in hand. In this connection the continuous study of toxin solutions which are allowed to stand for some time proved particularly instructive, for it was found that while the power to bind antitoxin remained constant, the toxicity gradually diminished. This study gave us one of the fundamental conceptions underlying the modern view of toxins, namely, that toxicity and combining power are two distinct and independent properties of the toxin molecule. As is well known, this fact is expressed by the side-chain theory by assuming that the toxin molecule possesses two specific atomic groups, one of which is toxophore, the other haptophore. Destruction or loss of the toxophore group gives rise to the non-toxic toxoids which are still capable of binding antitoxin. As a result of the high degree of lability of the toxophore group, this transformation into toxoid is a spontaneous process. And since the production of effective bacterial toxin solutions takes a certain time, it is obvious that we can practically never obtain a pure toxin consisting entirely of similar molecules. All our work must be done with toxic solutions which, even if we assume that the bacteria have produced only a single primary toxin, represent a mixture of toxin and toxoid.

But do the bacilli secrete only a single, homogeneous poison? This question has come more and more to be the subject of an animated discussion. Closely associated with it is the further question as to the nature of the reaction which occurs when toxin and antitoxin unite. The study of these problems was made possible by an important extension of quantitative toxin analysis, namely, Ehrlich's method of partial neutralization. This consists essentially in mixing a constant amount of poison with varying amounts of antitoxin and then determining the toxicity of the various mixtures, i.e., the decrease in toxicity brought about by each successive addi-

tion of antitoxin. By means of a graphic representation of the figures thus obtained, we can get a deeper insight into the details of the combining phenomena. Even now, after physical chemistry has taken such great interest in the reactions between toxin and antitoxin, all the various statements concerning the subject are finally based on the method of partial neutralization.

From the outset Ehrlich felt sure that toxin and antitoxin could not be simple substances of strong affinities which combined, for instance, like caustic soda and hydrochloric acid. This was evidenced particularly by the phenomenon which has often been termed the "inequality" of serum experiments. Thus if varying amounts of toxin are added to a constant amount of antitoxin (an immune unit), two distinct limits will be obtained:  $L_0$  (=Limit zero) is the quantity of toxin in which the mixture is just completely non-toxic, i.e., physiologically neutral.  $L_+$  (=Limit death) is the quantity of toxin in which the mixture is still just able to exert all its characteristic toxin action, i.e., in the case of diphtheria poison to just kill the guinea-pig acutely. Now if toxin and antitoxin behaved like caustic soda and hydrochloric acid, the difference between  $L_+$  and  $L_0$ , which we shall term  $D$ , should correspond to one lethal dose ( $L. D.$ ). As a matter of fact, however,  $D$  is usually considerably larger, so that our first inequality becomes  $L_+ - L_0 > L. D.$

Hence only two possibilities exist. Either toxin and antitoxin react with one another like a weak base and a weak acid (e.g., ammonia and boric acid), in which case the high value of  $D$  is the expression of an incomplete neutralization, or else the poison solution, besides the real toxin, contains a second substance of less affinity. This substance, while unable to produce the characteristic toxin effects, gives rise to certain mild toxic phenomena. In the case of diphtheria poison (owing to the practical importance of diphtheria antitoxin, the discussion has usually centered around this poison) human pathology had long taught that acute diphtheria infection is often followed by a second set of intoxication phenomena, namely, the peculiar paralyses which develop after the acute disease has disappeared. A priori, therefore, the assumption was highly probable that the high value of  $D$  was due to different components of the poison. And when the results of clinical experience and animal experiments harmonized so perfectly, the probability became almost a certainty. It has been found that the toxicity of mixtures whose toxin content lies between  $L_0$  and  $L_+$  is not quantitatively diminished, but is actually

different qualitatively. Guinea-pigs injected with such mixtures sicken, after a long period of incubation, with typical paralyses and show no local reaction. The hypothetical toxic constituent which gives rise to these paralyse sis termed "toxon."

Why then is it impossible to demonstrate the action of the toxon in native diphtheria poison? This is readily explained by the relative concentration of toxin and toxon in the toxic bouillon. Quantitative analysis has shown that the toxin is usually much more (about 5 times) concentrated than the toxon. Hence the fractional parts of the lethal dose which allow the animal to live long enough to manifest toxon effects usually contain too little toxon to produce the typical paralyses. If, however, a large amount of poison is so far neutralized with serum that all the toxin, with the higher affinity, is just bound and the toxon is still free, a mixture will be obtained which practically represents a pure toxon solution, for the neutral toxin-antitoxin molecules play no rôle in an animal experiment. It is at once apparent that, in view of the individual multiplicity of vital phenomena, the poisons of all strains of diphtheria bacilli will not contain both components in the same *relative* concentration. As a matter of fact, we find that the number of lethal doses contained in the difference  $L_t - L_0$  varies enormously, and so far as the toxon content is concerned the variations were from 0 to 300% figured on the basis of the toxin content. It will be well to enter somewhat more into a study of these two extremes, for these striking exceptions to the typical conditions argue strongly in favor of the views here presented. One of the poisons in question was studied by Ehrlich, and was remarkable in that the difference  $L_t - L_0$  represented only 1.7 lethal doses. We may therefore assume that the poison was free from toxon or nearly so, for the value of  $D$  was actually quite near one lethal dose, the figure demanded of a toxon-free poison, provided toxin and antitoxin combine like a strong base with a strong acid. The opposite extreme was manifested by a poison described by Dreyer and Madsen. The constants of this showed that it contained three times as much toxon as toxin. This poison, moreover, gave rise to toxon effects when sublethal dose of the native poison, without serum addition, were injected into animals. In view of what we have said above, this is readily understood, the relative concentration of toxon in this case was so great that even sublethal doses sufficed to make the toxon effects manifest. In most native poisons this demonstration fails because of the slight relative content of toxon.



The existence of the toxons which has been deduced mathematically from the biological experiments is, however, no longer based merely on these calculations. At the present time their existence is a proven fact, for quite recently van Calcar succeeded in separately isolating toxin and toxon from the native poison solution by means of a ingenious dialyzing procedure. Owing to its smaller molecular volume, toxin diffuses through a suitable membrane under less tension than toxon. In this way one obtains toxon-free toxin on one side and toxin-free toxon on the other.

This direct confirmation of the conclusions drawn from the biological analysis of the toxins shows how a mathematical study, provided biological facts are carefully regarded, can get at the nature of the phenomena in question, despite the failure of chemical methods. To be sure the mathematical treatment of biological problems must be undertaken very carefully. The phenomena of animate nature are so manifold, and subject to so much change, that they cannot all be forced into the limits of a formula. It is particularly dangerous to build up formulas and laws on the basis of too simple assumptions. For them one can easily be deceived by the apparent exactness of figures, and arrive at conclusions which do not sufficiently regard the complexity of the actual phenomena.

Unfortunately these warnings are much needed at the present time, for certain high authorities are striving energetically to explain the most complex phenomena, like those which occur in the union of toxin and antitoxin, as though they were simple and readily calculated reactions between simple substances.

In opposition to the plurality of the poison constituents demonstrated by Ehrlich, Arrhenius and Madsen, as is well known, uphold a unitarian standpoint. Their deductions are based entirely on the method of partial neutralization introduced into toxin study by Ehrlich and referred to above. Up to this point they differ only in the method of representing their results graphically. For this purpose they use a system of coordinates, laying off the amounts of antitoxin contained in each mixture on the abscissas. But whereas in Ehrlich's scheme the ordinates represent the amounts of toxin which each addition of antitoxin causes to disappear, Arrhenius and Madsen use the ordinates to represent the toxicity which each mixture still retains. In their work these authors observed that now and then in a number of poisons, especially in tetanolysin, the line connecting the points plotted possessed a certain similarity to curves obtained when weak

bases are neutralized by weak acids (ammonia and boric acid). This similarity constitutes the basis for their mathematical work, which leads them to conclude that toxin and antitoxin are simple substances whose reaction is reversible. This reaction finds its expression in the curve just mentioned. Let us examine their conclusions and see whether they are justified.

The two graphic methods referred to are equally correct. Nevertheless it cannot be denied that the one employed by Ehrlich, the so-called "poison spectrum," has certain advantages, for it brings out more clearly any deviations from the regular curve. Speaking mathematically we say that the "poison spectrum" is the graphic representation of the differential quotients of Arrhenius and Madsen's curve. In this sense, the ordinates of the spectrum represent the direction of the neutralization curve, i.e., the trigonometric tangent of the angle which the tangent forms at every point with the axis of the abscissas. Hence, if the course of the neutralization curve is that of a straight line, the direction therefore being the same at all points, we must represent the poison spectrum as a rectangle. If, as is often the case, the addition of a small amount of antitoxin causes no decrease in toxicity (prototoxoids), so that the neutralization curve in this part of its course lies parallel to the axis of the abscissas, we must represent the poison spectrum as having a gap at this point, for the angle between tangent and axis of abscissas is  $0^\circ$ . This brief statement should make it clear that in the poison spectrum, by representing the direction of the separate parts of the curve as ordinates, deviations from the regular curve-like course will be more clearly shown. It may be well to study these conditions by means of a diphtheria poison investigated by Madsen.<sup>1</sup> See Figs. 1 and 2.

These figures show that the deviations from the hyperbolic curve demanded by Arrhenius and Madsen's views are much more clearly shown in the representation employed by Ehrlich. Entirely aside from the question whether the sharply defined zones of the poison spectrum actually exist, or whether a gradual transition must be interpolated, it is certain that the changes should always occur in the same way; for they merely represent the differential quotients of the neutralization curve, and should therefore, if this curve were hyperbolic, show a successive decrease. The manifestly very irregular

<sup>1</sup> The sole object in employing this poison is to illustrate the two methods of graphic representation.

rise and fall of the differential quotients shows at once that a hyperbolic curve is out of the question in the case pictured above. If we examine the poison spectrum, on the other hand, we find that this represents Madsen's poison entirely in accord with Ehrlich's views concerning the constitution of diphtheria poison. If toxin and antitoxin unite firmly, and the course of the neutralization curve therefore is a straight line, the irregular course is explained by the toxoid present in the poison and by the varying affinity of the poison constituents. The highest zone in the poison spectrum (zone *c*) indicates that at this point equal amounts of antitoxin cause the greatest

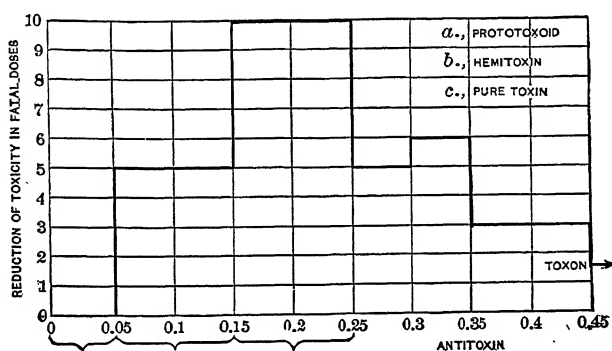


FIG. 1.—Poison spectrum according to Ehrlich.

decrease in toxicity. Hence this part of the poison must contain the least toxoids, or none at all, and we may therefore speak of this as pure toxin. It will serve as a unit for judging the degree of contamination with toxoid in the remaining portions. We should then speak of zone *b* as the hemitoxin, i.e., for each molecule of toxin there is one of toxoid. The sequence of the different zones corresponds to the different affinities of the components. Thus we see that the addition of a small amount of antitoxin (*a*) does not cause any decrease of toxicity whatever. And yet the antitoxin must have been bound. We conclude, therefore, that toxoids must here be present which possess a higher affinity than any other constituent of the poison. We are here dealing with the important prototoxoid zone which we encounter so frequently in diphtheria poison, abrin, ricin, crotin, etc. The hemitoxin zone which follows this is to be regarded as a deuterotoxin in its affinity. The constituents of the

poison can thus be arranged as proto-, deuto-, —, tritotoxin, etc., after which finally comes the constituent possessing the weakest affinity, namely, the toxon. That this varied affinity does not arise when the toxoids are formed, but differentiates the undecomposed constituents of the poison from the outset, is demonstrated by the genesis of toxoid formation. Thus if one is in a position to study a very pure poison in its various stages of decomposition, it will be found that there is a first phase which leads to the formation of hemitoxin, and that a later phase changes this into prototoxoid. If there were a change in affinity, however, we should have had a pure toxoid zone from the start.

The prototoxoids proved a serious obstacle to Arrhenius and Madsen in the logical development of their views. According to their theory just the first amounts of antitoxin added should decrease the toxicity the most. Nevertheless a number of experiments were published by these authors (Madsen, with diphtheria poison, and Madsen and Walbaum, for ricin) in which the prototoxoids and their development were only too apparent. And Arrhenius and Madsen seem to appreciate that they can no longer explain this contradiction by assuming that the prototoxoid zone is due to "change-ments minimes dans le milieu ambiant," or by saying that the proto-

toxoid zone is "of little interest." In order, therefore, to eliminate these prototoxoids, so annoying for their formula, they have discarded the well-tried criterion for a fatal dose of diphtheria poison (death of the guinea-pig in 3 to 4 days), and now attempt to calculate the fatal dose in a new way. Their procedure is as follows: Retaining the definition of a fatal dose, they believe it possible to calculate the fraction or multiple of the fatal dose employed, from the time of the animal's death or even from the resulting loss of weight. Such

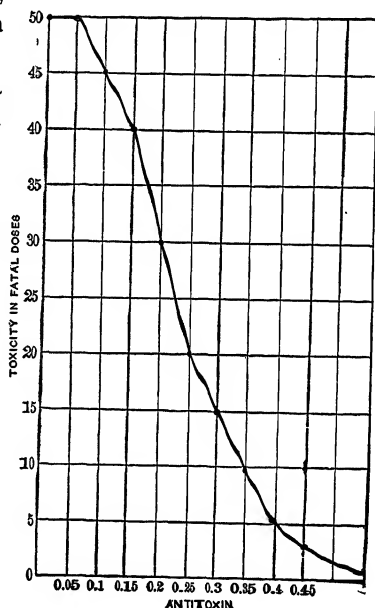


FIG. 2.—Neutralization curve according to Arrhenius and Madsen.

a procedure, in order to possess any justification whatever, would have to be based on an enormous experience. But even aside from this it is amazing to see how a lot of experimental protocols, going back to 1897, are unhesitatingly used for their calculations. The old determinations of the lethal dose, in which death produced acutely in 3 to 4 days was the criterion, are very difficult to make use of owing to the individual variations in the animals. Certainly it requires some experience to know which animals should be discarded because of over- or undersusceptibility. But how much more complex the conditions really are is at once apparent if one attempts to determine  $\frac{1}{2}$  or  $\frac{1}{3}$  of a lethal dose from the clinical course of the disease. Hence it is not surprising to find that the lethal doses calculated by Arrhenius and Madsen represent the averages of figures which often differ from each other by many times. The tedious work which these authors have undertaken may perhaps satisfy a mathematician; to the biologist, however, it can only represent useless and dangerous playing with figures. It signifies nothing, therefore, if the figures recently obtained by this method by Arrhenius and Madsen with three poisons fail to show any prototoxoid zone.<sup>1</sup> For the same reason, also, we cannot regard certain other figures, which differ markedly in observation and calculation, as arguments against their views.

However, we need neither confirmation nor controversion of their theory. For it has been found that the assumptions on which this theory is based have no existence whatever. We have already alluded to the fact that van Calcar has recently demonstrated the existence of toxons. But it has also been shown by another method that diphtheria poison, as well as most other toxins, must contain various constituents capable of binding the antitoxin. This method had its inception in the following considerations.

Arrhenius and Madsen, as already stated, regard the union of toxin and antitoxin as a reversible reaction between two simple [*einheitlich*] substances. According to this view, therefore, the reaction is incomplete, i.e., the two substances reacting (toxin and antitoxin) are never completely used up, a certain portion of both toxin and antitoxin always remaining free beside the neutral toxin-antitoxin combination. The equilibrium which exists between the three components

<sup>1</sup> We should not neglect to mention that the existence of the prototoxoid zone and its development from the hemitoxin phase has also been demonstrated in diphtheria poison by so excellent a worker as Theobald Smith.

will then be governed by the law of mass action formulated by Guldberg-Waage, namely,  $(\text{toxin}) \cdot (\text{antitoxin}) = k(\text{toxin-antitoxin})$ , in which the brackets denote the concentration, and  $k$  the constant of equilibrium to be determined for each poison.<sup>1</sup> All the calculations of Arrhenius and Madsen are based on this formula, and their entire work stands or falls with the applicability of the formula to the subject of toxins.

The formula, however, is only then applicable if the reaction is really completely reversible, and this is not the case. Thus if mixtures containing the same amounts of toxin and antitoxin are tested at the end of the reaction, it is easy to convince one's self that the toxicity is dependent not only on the amounts of toxin and antitoxin, but on the manner of making the mixtures. If to the same amount of antitoxin we add at intervals fractional parts of the toxin, we shall find that the resulting end product is considerably more toxic than if the same amount of toxin is mixed with the antitoxin at once. This holds true even if the toxin is added at the time corresponding to the addition of the last fraction in the former case. Von Dungern was the first to point out the significance of this experiment, in connection with an observation made by Danysz, for the question of reversibility. He showed that if this really was a completely reversible reaction between simple substances, as is assumed by Arrhenius and Madsen, we should expect that the same equilibrium should always ensue with the same total amounts of reacting substances, i.e., the toxicity of the end products should always be the same. Any deviation from this could occur in the fractioning process only during the course of the reaction; and then, provided the deviation were a function of the reaction-time, this would be just the reverse of what is actually observed.<sup>2</sup> Hence all those poisons in which this phenomenon of

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<sup>1</sup> In their recent publications Arrhenius and Madsen assume that one molecule toxin combines with one molecule antitoxin, not to form two molecules of the toxin-antitoxin combination, as the above formula would show, but that two different substances are formed, toxinan and titoxin. To be sure as the equation then reads,  $(\text{toxin}) (\text{antitoxin}) = k (\text{toxinan}) (\text{titoxin})$ , one objection to the above formula is done away with, but a new hypothesis, lacking all evidence whatever, is thus introduced merely for the sake of the formula.

<sup>2</sup> The phenomenon in question therefore shows exactly the reverse of what Arrhenius and Madsen's theory demand. For this reason the limit of error need not be considered, although, owing to the enormous quantitative differences, it would play no rôle in judging the result. Nor can Arrhenius extricate himself from the predicament by suggesting that we are dealing with slowly progress-

increasing toxicity on the fractional addition of toxin can be demonstrated must at once be excluded from any mathematical analysis based on a formula of equilibrium derived from the law of mass action. In all of the cases<sup>1</sup> examined for the purpose (diphtheria poison, tetanolysin, ricin, staphylolysin, arachnolysin, rennin, and precipitin), this method has shown that the conception of Arrhenius and Madsen is entirely inapplicable.

The phenomena observed, however, are very readily explained by the assumption of a plurality of combining groups in the poison solution. Thus if to an excess of antitoxin a small quantity of poison is added, as is done in the fractioning experiment, the result would be that even the constituents possessing a feeble affinity and which are of no consequence so far as any toxic action is concerned, would be bound by the antitoxin. When then the second portion of poison is added, it will be impossible for the toxin molecules, although possessing a higher affinity, to crowd the previously bound constituents out of their combination with the antitoxin. The result is that a certain portion of toxin, which would have been neutralized by the antitoxin if all the poison had been mixed with the antitoxin at once, now remains free. That is to say, the fractional method of adding the poison has resulted in an increased toxicity, the  $L_+$  dose being reached with a smaller amount of poison. Furthermore it is possible, by means of suitable technique, to cause a reduction of the  $L_0$  dose, from which it follows that the  $L_0$  serum mixture contains free non-toxic constituents capable of binding antitoxin, and that these must possess still less affinity than the toxon. These are the so-called "epitoxonoids" of von Dungern. The discovery of the epitoxonoids also offers an easy explanation of the fact that it is possible to immunize with mixtures of toxin and antitoxin which are physiologically neutral.

All this shows that a complete reversibility, even of the individual

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ing side reactions which do not interfere with the main reaction when one works rapidly. For, as was pointed out by von Dungern and Sachs, the increased toxicity is already demonstrable at a time when the union of toxin and antitoxin is not yet ended. The hypothetical "side reaction" would therefore proceed just as quickly as the main neutralizing reaction.

<sup>1</sup> The single exception met with, namely cobra venom, only proves the rule; for cobra venom (we are dealing with the hæmolytic portion which is activated by lecithin) is a simple substance with a strong affinity for the antitoxin, as can be seen from the course of the neutralization curve, which is a straight line.

poison constituents, is out of the question. On the contrary we must assume that the union of these substances with the antitoxin is subsequently tightened. This tightening is also borne out by other observations, both old and recent. If the toxin-antitoxin reaction were reversible, it should be possible, by removing the supposedly free toxin residue, to constantly change the equilibrium, so that the toxin could all be recovered. Nevertheless, although toxin can be filtered through gelatine and antitoxin cannot, it is impossible either by gelatine filtration (Martin and Cherry) or by gelatine diffusion (van Calcar) to obtain free toxin from neutral toxin-antitoxin mixtures.<sup>1</sup> In addition to this one cannot help being surprised that the calculations of Arrhenius and Madsen entirely ignore the cells' toxin-binding receptors which effect the poisoning. In accordance with their views, these receptors should represent an important element in the equilibrium; and yet they appear to have entirely overlooked this fact.

It would lead us too far to discuss all the arguments against the views of Arrhenius and Madsen. It will suffice to call attention to the serious objections which Nernst has raised regarding the principles involved, and to Köppe's criticism of their technique in making hæmolytic test-tube experiments. This illustrates the danger of a one-sided mathematical study of biological problems. Even if one succeeds now and then in making the figures of observations and calculation tally, it is impossible at the present time for these mathematical expressions to explain the facts. To be sure they may be able to represent the resultants of the processes which bring about the phenomena, but in that case the formula is nothing more than an interpolation formula. Corresponding to this, therefore, we see that the formulas of Arrhenius and Madsen vary widely for the same poison, every new lot of poison of the same bacillary origin has a new constant of equilibrium. Hence the formula is applicable only to one particular case, and so, even if it were a correct interpolation formula, progress of biological science would in no way be furthered by it.

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<sup>1</sup> It is perfectly evident that toxin can be obtained from fresh toxin-antitoxin mixtures by diffusion through gelatine, and this has recently been demonstrated by Madsen and Walbaum. According to Morgenroth such mixtures require at least twenty-four hours for the union to become complete. Hence the statement by Madsen and Walbaum that the mixtures must be fresh in order to demonstrate what they regard as dissociation only confirms our view.



Biology does not content itself with a mere registration of phenomena; it seeks to discover their nature and their relation to one another. In fact the chief mission of biology is to attempt, by linking facts and theories and hypotheses, to satisfy the craving of the thinking naturalist for an insight into causes.

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## THE MECHANISM OF THE ACTION OF ANTIAMBOCEPTORS.<sup>1</sup>

By Prof. PAUL EHRLICH and Dr. H. SACHS.

to closer investigations into the nature of immunity our  
s regarding the relation between antibody and the sub-  
citing the production of immunity (the antigen, as it is  
ve undergone a certain modification. This consists in a  
ise definition of the concept specificity. In the beginning  
umed that an antibody produced by immunization acted  
st the substance through which it was developed. Further  
is, however, soon brought to light cases in which this  
pparently violated. A clear insight into this subject was  
de possible when the receptor was looked upon as the  
sh excited the production of immunity. According to  
ain theory, therefore, specificity of the antibodies always  
e *specific relations between the individual types of antibodies  
ctors.*"<sup>2</sup> Since, therefore, the same receptor can be dis-  
t only among different kinds of cells, and bodies of differ-  
ns all within the same animal species, but also among  
pecies of animals, we see that it is impossible to speak of  
r in a zoological sense, or of a specificity in respect to the  
cal or functional properties of the antigens. The anti-  
cific only for the receptor, i.e., for those elements possess-  
ing receptor.

various substances which excite the production of im-  
pecial place is occupied by the receptors of the third order:  
1 free, constitute the amboceptors. As is well known,  
ptors possess a double function. On the one hand they  
the cytophile group of the cells, and on the other with the

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Printed from Berliner klin. Wochenschrift, 1905, No. 19.  
Ehrlich and Morgenroth, Hæmolysins. See page 88.

complementophile group of the complement. Each of these two haptophore groups will therefore be able to excite the production of corresponding antibodies, a fact to which attention was called in the Croonian lecture, 1900.<sup>1</sup>

"The lysin, be it bacteriolysin or hæmolysin, possesses altogether three haptophore groups, of which two belong to the immune body and one to the complement. Each of these haptophore groups can be bound by an appropriate antigroup." Three 'antigroups' are thus conceivable, any one of which, by uniting with one of the haptophore groups of the lysin, can frustrate the action of the lysin."

In other words, according to the amboceptor theory two different antiamboceptors are at once conceivable, either of which would inhibit the action of the amboceptor. One would act by preventing the union of amboceptor and cell, the other by preventing the complement from uniting with the amboceptor. Originally the antiamboceptors produced by immunization were regarded as being directed against the cytophile group.<sup>2</sup> In view of this it was extremely desirable for the support of the amboceptor theory that the existence of antibodies for the complementophile group should be demonstrated. This has recently been done by Bordet,<sup>3</sup> and it is strange to see that he employs his discovery in combating the receptor theory when it really is a very neat confirmation of this.

Bordet finds that antiamboceptors can be produced not only by immunization with hæmolytic immune serum, but also with normal serum of the same species, even though this normal serum contains no corresponding amboceptors. He treated guinea-pigs with normal rabbit serum which contains no hæmolytic amboceptors for ox blood, and obtained an immune serum which yet was able to inhibit the action of the amboceptors derived by immunizing with ox blood. That, certainly, is a discovery which cannot readily be explained in harmony with Bordet's sensitization theory. According to Bordet, as we know, these immune bodies (his "sensitizers") possess the one property of combining with the susceptible cell and thus rendering this vulnerable to the action of the complement. This being the case it is incomprehensible how a serum which possesses

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<sup>1</sup> P. Ehrlich, *On Immunity*, Proceedings Royal Society, 1900.

<sup>2</sup> Ehrlich and Morgenroth, VI. Communication, page 88.

<sup>3</sup> J. Bordet, *Les propriétés des antisensibilatrices et les théories chimiques de l'immunité*. *Annal. de l'Institut. Pasteur*, 1904, No. 10.

no sensitizers whatever for the species of cell in question can yet excite the production of antibodies directed against them. The matter takes on an entirely different aspect if we regard this phenomenon from the standpoint of the amboceptor theory. According to what has been said above we at once see that two functionally different types of antiamboceptors are possible. In Bordet's case the normal rabbit serum possessed no amboceptors (i.e., no cytophile groups) for ox blood; therefore the antibodies which are developed cannot be antiamboceptors directed against the cytophile groups. Hence by exclusion one will already pronounce them *anti-amboceptors of the complementophile group*. The facts brought forward by Bordet all go to confirm this.

If such antiamboceptors are to be produced, the only requisite is that the serum used for immunization must contain the corresponding complementophile groups. Is this the case in normal rabbit serum? Every normal rabbit serum, as Bordet admits, contains a large number of different amboceptors. If, by immunizing with a given species of cell, a new specific amboceptor develops in the serum, *the new element in the receptor apparatus is really only the cytophile group*, which is produced in response to immunization. The complementophile apparatus need not suffer the least change qualitatively; in fact according to our conception it usually does not change markedly, there is merely an increase in the complementophile groups corresponding to the formation of the additional immune body. We have already expressed this opinion in a previous paper.<sup>1</sup> "In my judgment we shall arrive at a correct conception if we proceed from the standpoint that in general the specific amboceptors exhibit a uniform structure so far as their complementophile portion is concerned, while their cytophile groups, which physiologically are concerned with the absorption of food, differ most widely."

It must not be thought that this uniform constitution of the complementophile portion<sup>2</sup> contradicts the assumption of a multiplicity

<sup>1</sup> P. Ehrlich, Betrachtungen über den Mechanismus der Amboceptorwirkung und seine teleologische Bedeutung. Koch Festschrift, Jena, 1903.

<sup>2</sup> For the present we cannot say whether the complementophile complex is really uniform throughout or whether, perhaps, certain partial groups do not differ in the individual amboceptor types of the same animal species. Such a condition is easily conceivable. In any event we must assume that the complementophile apparatus of the amboceptors of a given species is identical at least in some essential part of its haptophore functions, and that this characterizes it as coming from the animal species in question.

of complements. Naturally the different complements must have different complementophile groups corresponding to them. But, as was stated in the Sixth Communication on Hæmolysins,<sup>1</sup> an immune body, in addition to a particular cytophile group, contains two, three, or more complementophile groups. In a later paper Ehrlich and Marshall offered experimental evidence for just this point; besides this, Bordet's experiments, according to which an amboceptor after having combined with cellular elements is able almost completely to rob a serum of its complement, also support this view.<sup>2</sup>

We must therefore conceive the amboceptor to be structurally a polypeceptor, and assume further that the amboceptors of a distinct species are all supplied with a large number of complementophile groups which vary considerably in detail but in their entirety represent a uniform complex. This complex is reproduced in all the amboceptors of the same serum. In general the amboceptors are different and specific only so far as the cytophile group is concerned.

This being so it will at once be clear that antiamboceptors directed against the complementophile groups, and obtained through immunization with any particular amboceptor, will act against all amboceptors of the same animal species no matter whether these amboceptors are normally present in the serum or have been produced by immunization. For the complementophile amboceptor apparatus is the same for all types of amboceptors of the same species. As a result of this, an immune serum obtained through immunization with normal serum contains, thanks to the normal amboceptors in the serum, antiamboceptors directed against the artificially produced amboceptors of the same species. This explains also the earlier observations made by Pfeiffer and Friedberger<sup>3</sup> that antiamboceptors obtained by immunizing with cholera serum act also against typhoid serum;<sup>4</sup> it also explains the recent experiments made by Bordet. We

<sup>1</sup> Ehrlich and Morgenroth. See page 88.

<sup>2</sup> P. Ehrlich and H. T. Marshall, Über die complementophilen Gruppen der Amboceptoren. Berl. klin. Wochenschr. 1902, No. 25.

<sup>3</sup> R. Pfeiffer and E. Friedberger, Weitere Beiträge zur Frage der Antisera und deren Beziehungen zu den bacteriolytischen Amboceptoren. Centralblatt f. Bacteriol. 1904, Vol. 37; also 1903, Vol. 34.

<sup>4</sup> Naturally the statement made by Ehrlich and Morgenroth (Berl. klin. Wochenschr. 1901, No. 21) that "it seems improbable, unless in a given case a fortunate coincidence intervenes, that anti-immune bodies will be obtained directed against the bactericidal immune bodies" cannot apply to the antiamboceptors directed against the complementophile groups. That statement applies

must call particular attention to the fact that the chief point in Bordet's study, the non-specificity of the antiamboceptors so far as the cytophile group is concerned, had already been published by Pfeiffer and Friedberger. These authors have explained the fact entirely in accordance with our views, as follows:

"We are inclined to believe that the various immune bodies of one and the same animal species possess one group in common which in a way stamps them as coming from that particular animal organism. The antiserum must possess certain relations to this group." To this we would add that for the present it seems simplest to class this group or groups, specific for the animal species, with the complementophile group. In the amboceptor we differentiate a specific cytophile group and a large apparatus made up of complementophile groups. Aside from the property of anchoring the cells, the latter groups exercise all the remaining functions of the amboceptor. Considering that the normal amboceptors and those produced by immunization are essentially similar (a point which we have always emphasized), it is perfectly obvious that one can produce the same antiamboceptors by immunizing with normal amboceptors. Hence what Bordet's study really brings forward is the actual experimental demonstration of what we had long expected was the case.

Naturally we were able to confirm all of Bordet's statements of fact. We had at our disposal the serum of a goat which had been immunized with normal rabbit serum, and could easily convince ourselves that this serum acts as an antiamboceptor against amboceptors derived from rabbits by specifically immunizing with ox blood. Furthermore, we succeeded, by adding the antiamboceptor to previously sensitized blood-cells, to protect these against hemolysis by complement. The antiamboceptor acts just like a complementoid according to the conception of "complementoid-blocking" described by one of us some time ago.<sup>1</sup> It occupies the complementophile groups and so prevents the anchoring of the complement.<sup>2</sup>

only to the antibodies directed against the cytophile groups, since it is to be assumed that these cytophile groups, which have their natural counter-groups in bacterial cells, will not have these in the cells of higher animals. This limitation, however, does not apply to the antiamboceptors acting on the complementophile complex. This, then, disposes of Bordet's objections to this point.

<sup>1</sup> Ehrlich and Sachs, "Über den Mechanismus der Amboceptorenwirkung. Berl. klin. Wochenschrift, No. 21, 1902.

<sup>2</sup> We must not fail to mention that, in contrast to Bordet, we made these experiments without the addition of inactive guinea-pig serum, and were able, despite

We were also able to readily confirm Bordet's statement that the antiamboceptor action is easily inhibited by normal rabbit serum. Naturally the normal amboceptors, whose complementophile groups excited the production of the antiamboceptor, will combine with this antiamboceptor and so be able to deflect it from the amboceptor acting in the given case. Since we regard the antiamboceptor in the sense of a complementoid, this phenomenon corresponds in principle to that described by Neisser and Wechsberg as deflection of complement.<sup>1</sup>

The entire complex of phenomena just discussed shows most strikingly that our assumption harmonizes best with the observed facts. We assume that in Bordet's antiamboceptors we are dealing with antibodies directed against the complementophile groups. The existence of such antiamboceptors again demonstrates that the amboceptor theory is correct. According to Bordet's sensitization theory only such antiamboceptors are conceivable which prevent the amboceptor's union with the cell. But if there are other kinds of antiamboceptors, as the findings just discussed show, we must assume that the amboceptor has other affinities besides those for the cell, and this leads us at once to the conception which we have defined under the name amboceptor. The sensitization theory must therefore be abandoned.

The next question which arises is whether or not it is possible by means of immunization with amboceptors to produce antiambo-

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this, to effect an inhibition of hæmolysis by subsequently adding antiamboceptor. It seems to us that this simplified procedure is more convincing, for it will hardly be claimed that the guinea-pig serum is a better suspending medium than physiological salt solution, and that it therefore, in contrast to the latter, leaves the blood-cells intact. Furthermore, inactive guinea-pig serum itself inhibits the hæmolysis of ox blood by amboceptor and complement (guinea-pig). Hence when guinea-pig serum is present the question whether the absence of hæmolysis is due to an antiamboceptor or not is left undecided.

<sup>1</sup> In contrast to Bordet, however, we were unable by means of normal amboceptor to effect the subsequent breaking of the union between antiamboceptor and sensitized blood-cells. It may be that in our case the union between antiamboceptor and sensitized cells so rapidly became firm that it could no longer be dissolved by the normal amboceptor. Even Bordet admits that this dissolution can be effected only for a certain period, and that then the union becomes very firm. We are pleased to note that Bordet accepts this conception of a gradual tightening of the union of these substances, a conception of the highest importance in the study of immunity reactions.



ceptors also against the cytophile group. We have therefore examined another antiamboceptor serum, and compared its properties with those of the antiserum made by injections of normal rabbit serum. This serum, like the latter, was also obtained from a goat, but instead of using normal rabbit serum for immunization the goat had been treated with the serum of a rabbit previously immunized with ox blood. Our experiments, however, did not permit of a decision on this point. We are unable to say whether among the antiamboceptors excited by the injections of the immune serum there were any directed against the cytophile group. It is entirely conceivable that, despite the presence of the cytophile group, these are unable to exert any immunizing power, since the complementophile groups invariably encounter the corresponding counter-group in the organism and so are the only ones bound to the tissue receptors. In that case previous to injection one would attempt to destroy the complementophile group (=cytophilic amboceptoids) or to neutralize it by means of a suitable antibody. The decision of this question must be left to further detailed investigations.

In the course of our experiments we met with a very curious phenomenon, one not only of some practical significance, but also of considerable theoretical interest. Our experiment showed exactly the opposite behavior which Bordet had found. That is to say, where Bordet found that the antiserum acts as an antiamboceptor on the amboceptor anchored to the cell, and that this action is overcome by normal rabbit serum, one of our cases represents the reverse of this. We see, therefore, that it can happen that the antiamboceptor as such does not act, but requires the addition of normal rabbit serum before exerting its action. We have constantly observed that in a "curative" experiment, i.e., after a previous binding of amboceptor and cell, large amounts of the antiserum produced by means of immune serum were unable to prevent hæmolysis. The following protocol may serve as an example:

To each of a series of test-tubes, containing decreasing amounts of the antiserum, 1 cc. of ox blood was added. This blood, after having previously been sensitized with 0.003 cc. ( $=1\frac{1}{2}$  amboceptor units) of an amboceptor obtained from a rabbit by immunization with ox blood, was freed from serum constituents by centrifuging and then used in the test. After digesting the mixtures for half an hour the blood-cells were centrifuged off and the sediments, to which 0.1 cc. guinea-pig serum was added as complement, were

suspended in salt solution. The result of the experiment is shown in the following table:

TABLE I.

Amount of the Antiserum (derived from a goat by treatment with an am- boceptor, the result of immunizing a rabbit with ox blood) cc.	Amount of Hæmolysis.
0.1	complete
0.05	well-marked
0.025	moderate
0.015	little
0.01	0
0.005	faint trace
0.0025	very little
0.0015	moderate
0.001	almost complete
0.0005	complete
0.00025	complete
0	complete

Here we see the curious result that with a certain excess of the antiserum there is no inhibition of hæmolysis. This paradoxical phenomenon we observed only with the antiserum produced by immune serum injections, and then only in the "curative" experiment. If the antiserum was used for "protective" experiments, i.e., mixed with amboceptor previous to adding the blood-cells, or if the antiserum produced by injections of normal serum was employed, the course of the experiment was entirely uniform, an increase in the amount of antiserum causing an increase in the antilytic action. For the present we are unable to say whether we are here dealing with an essential difference between the antiserum produced by normal serum and that produced by immune serum, or whether we have to do with an individual fluctuation. So far as the mechanism of the phenomenon is concerned we were able to clear up at least one point, namely, that the essential factor in the experiment is the presence or absence of the very small quantities of normal rabbit serum which contains the amboceptor. Thus if the blood-cells are sensitized with amboceptor without subsequently removing the serum by centrifuging, it will be found that the course of the "curative" experiment is perfectly regular. There is no inhibition of the antilytic action with an excess of antiserum. The same holds true if we sepa-

rate the sensitized blood-cells by centrifuge and replace the serum fluid with the corresponding amount of normal serum (in our cases 0.003 cc.). The active substance contained in normal serum is thermostable at 56° C., but is destroyed by heating for half an hour to 100° C. The following experiment may serve as an illustration:

The blood-cells which have been sensitized with 0.003 cc. serum and then separated by centrifuge are treated with a considerable excess (0.5 cc.) of the antiserum. This amount corresponds to that quantity which by itself is just able to overcome the antilytic action. To this mixture are added decreasing amounts of normal rabbit serum which has been heated to 56° C. and to 100° C. After allowing the mixture to stand for half an hour the blood-cells are centrifuged off and suspended in salt solution to which 0.1 cc. guinea-pig serum (complement) is added.

The result is shown in the following table:

TABLE II.

Amount of Normal Rabbit Serum.	1 cc. 5% Ox Blood (sensitized with 0.003 cc.) + 0.5 cc. Antiserum + Normal Rabbit Serum.	
	a. Heated to 56° C. Amount of Hæmolysis.	b. Heated to 100° C. Amount of Hæmolysis.
0.005	0	} complete
0.003	0	
0.0015	little	
0.001	moderate	
0.0005	complete	
0	complete	

This shows us what a tremendous effect the presence or absence of a small amount of normal serum can exercise. This of course at once explains the difference which manifests itself between the "curative" and the "protective" experiments. In the latter, it will be recalled, the amboceptor and antiamboceptor are first mixed. All of the normal serum constituents, therefore, come into action; whereas in the "curative" experiment these are removed when the blood-cells are centrifuged.

How are we to conceive the mechanism of this action? Phenomena in which an excess of a certain substance produces a change in the character of the reaction are frequently due to the

presence of other substances with different properties. In the case described above there is an absence of antilytic action with a certain excess of the antiserum. If we look at the subject from this standpoint, we shall have to assume that the antiserum contains two substances,<sup>1</sup> one of which, of course, is the effective antiamboceptor. The other substance would then be the cause of the inhibition of the antiamboceptor action. Furthermore, since this inhibition is only brought about by large quantities of the serum, this substance would be present in the serum in much smaller amounts than the former. The simplest explanation of the action of this substance seems to be somewhat as follows: We must assume that this substance's point of attachment is a complementophilic auxiliary group in the amboceptor. The occupation of this group so affects the amboceptor molecule that the simultaneous presence of antiamboceptor no longer prevents the combination with complement. Such a behavior would be analogous to an observation published by Ehrlich and Marshall.<sup>2</sup> At that time, by means of a differentiating method made available for one particular instance<sup>3</sup> by Marshall and Morgenroth, it was shown that the amboceptor anchored to the cell, although it could deprive native guinea-pig serum of all its complement functions, was unable to absorb the non-dominant complements if the dominant complement had first been neutralized by the partial anticomplements of Marshall and Morgenroth. In other words, an anchoring of the non-dominant complements was only possible after the corresponding complementophile group of the amboceptor had combined with the dominant complement. In our case we would be dealing with an influence entirely similar in principle, except that here the influence is reversed, i.e., the affinity of the amboceptor to the antiamboceptor is reduced by the occupation of the auxiliary group. We believe that we can show directly that the antiamboceptor is bound in either case, but that where the auxiliary group is occupied, the union of amboceptor and antiambo-

<sup>1</sup> We can of course assume a priori that an antiamboceptor serum directed against the complementophile groups will possess a multiplicity of partial antiamboceptors, for the amboceptors which take part in the immunization possess a large number of different complementophile groups, and against each of these a particular antibody is conceivable.

<sup>2</sup> Ehrlich and Marshall, l. c.

<sup>3</sup> H. T. Marshall and J. Morgenroth, *Über Differenzierung von Complementen durch ein Partialanticomplement*. *Centralblatt f. Bact.* 1902, Vol. 31, No. 12.

ceptor remains a loose one, while in the other case it becomes firm. The following diagram may help to make this clear. See Fig. 1.

We shall designate the two complementophile groups of the amboceptor as  $\alpha$  and  $\beta$ ; the effective antiamboceptor corresponding to group  $\alpha$  is  $a$ , the antibody fitting group  $\beta$  is  $b$ . In small quantities of antiserum,  $b$  can practically be disregarded owing to its slight concentration;  $a$  therefore by occupying  $\alpha$  prevents the complement uniting with the amboceptor. In larger quantities of antiserum, however,  $b$  comes into play, so that the occupation of group  $\beta$

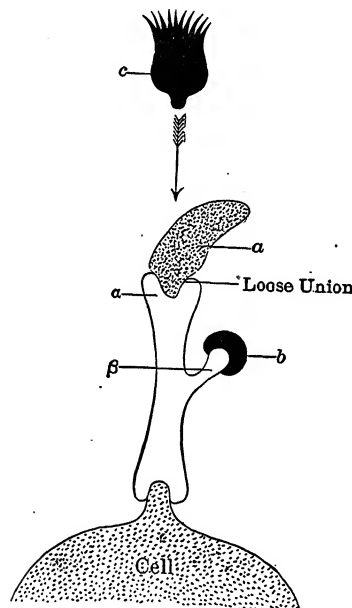


FIG 1.— $\alpha$  and  $\beta$ : Complementophile groups of the amboceptor.  $a$  and  $b$  are Partial Substances of the Antiserum.  $a$  is the effective Antiamboceptor;  $b$  is the antibody which inhibits the action of the antiamboceptor.  $c$  is the Complement.

changes the reactive capacity of group  $\alpha$  in such a way that either  $a$  is not bound at all while the corresponding complement is, or so that, while  $a$  may still be bound, the union is such a loose one that the complement still has access. We shall see that the latter possibility is the more probable. First, however, it will be necessary for us to understand clearly the manner in which normal rabbit serum overcomes the influence of the antiserum constituent  $b$ . In view of what has been said this will not be difficult, for it is but a

natural consequence for us to assume that normal rabbit serum contains the corresponding counter-group  $\beta$  in such high concentration that even small amounts are able to neutralize  $b$  and so prevent its union with the amboceptor anchored by the cell. See Fig. 2.

Coming now to the question whether, after group  $\beta$  is occupied, group  $\alpha$  no longer reacts with  $a$ , or whether, while the reaction takes place, the union remains a very loose one, we decided this according to the following considerations. If the latter assumption were correct, it would follow that the loose union should subsequently become

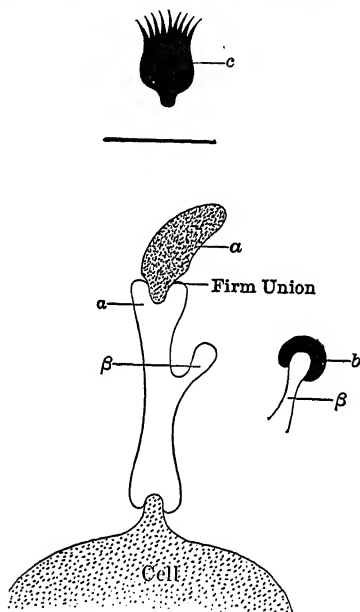


FIG. 2.— $\beta$ : Complementophile group of an amboceptor of normal serum. Otherwise as in Fig. 1.

firm if in some way group  $b$  could again be freed from its combination with  $\beta$ . In that case, evidently, the "curative" action of the anti-amboceptor  $a$  should become manifest. If, on the contrary,  $a$  has not been bound at all, this "curative" action should fail to appear on the removal of  $b$ .

Owing to the presence of group  $\beta$  in small amounts in normal rabbit serum the possibility is given of abstracting the antigroup  $b$  already bound to the sensitized cell. We have at once taken advantage of this fact, and attacked the question experimentally as follows:

Sensitized blood-cells are digested with an excess of the antiserum

(0.25 cc.). After centrifuging, decreasing amounts of inactivated normal rabbit serum are added to the sediments, and the mixtures again centrifuged. The blood-cells thus separated are suspended in 0.1 cc. salt solution containing 0.1 cc. guinea-pig serum. The result is shown in the following table:

TABLE III.

In active Normal Rabbit Serum. cc.	Amount of Hæmolysis.
0.01	} little to moderate
0.006	
0.003	
0.0015	
0	complete

This table, therefore, shows that sensitized blood-cells which have been treated with an excess of antiamboceptor and then freed from all free serum constituents by centrifuging can be deprived of a considerable portion<sup>1</sup> of the antiserum constituent *b* by subsequently digesting them with small amounts of normal rabbit serum, thus again allowing the antiamboceptor action to become manifest. It is permissible, therefore, to assume that the antiamboceptor *a* had been bound and that the union had remained a loose one owing to the occupation of group  $\beta$ . Owing to the looseness of the union *a* and  $\alpha$  the complement was not prevented from combining with the amboceptor.

We have gone into the analysis of this case with such detail because it again shows how complicated is the mechanism of amboceptors and yet how easy it is by means of the amboceptor theory to bring these apparently paradoxical phenomena into harmony. In this case we are certainly dealing with extraordinarily complex conditions, conditions in which Bordet's rudimentary sensitization theory is entirely helpless.

The phenomenon just described possesses a certain practical significance in so far as it could easily lead to the erroneous assumption

<sup>1</sup> It is likely that the reason why the inhibiting action cannot be entirely brought out by this means is that the union of *b*, once it is bound, rapidly becomes firm, thus permitting only a partial dissolution by means of free  $\beta$ . In any event this experiment clearly exhibits, as already stated, exactly the reverse behavior of that shown by Bordet's.

tion that the antiamboceptor acts only in "protective" experiments, but is unable to act on amboceptor already anchored by the blood-cells. In order to orientate ourselves concerning this last question, we would of course begin by using an excess of antiamboceptor, expecting very naturally, if the antiamboceptor exerts any influence whatever on the anchored amboceptor, that this influence will most likely become manifest with large amounts of antiamboceptor. Furthermore, it can then happen that the conditions obtaining are those of the zone in which the curative action obtained with smaller doses is concealed, owing to the excess of antiamboceptor. This may perhaps account for Morgenroth's negative findings;<sup>1</sup> the antiamboceptor serum employed by us was also used by that author.

The demonstration of the fact that the antiamboceptors produced by immunization are usually directed against the complementophile groups calls for a correction of certain deductions based on our earlier conception of antiamboceptors as being directed against the cytophile group. We must therefore concede that Bordet is correct when he refuses to accept our method of differentiating partial amboceptors by means of antiamboceptors, a method which we published in the Sixth Communication on Hæmolytins.<sup>2</sup> Our experiments at that time dealt with an amboceptor of an immune serum derived from a rabbit by treatment with ox blood. This amboceptor could be complemented either with guinea-pig serum or goat serum. In complementing with goat serum so much more amboceptor is necessary that the absence of the antiamboceptors' action must be ascribed to the antiantilytic action of the normal amboceptors present. But this correction does not signify that the conclusion as to the plurality of the amboceptors must be abandoned. On the contrary this conclusion is confirmed by so many weighty arguments of a different kind that the existence of partial amboceptors must now be classed as one of the facts in immunity. We need only call attention to a point contained in our Sixth Communication, namely, that by mutual elective absorption we have shown that immunization of animals with ox blood results in the formation of two fractions of amboceptors, one of which acts only on ox blood, the other also on goat blood; and that immunization with goat blood has exactly analogous reverse

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<sup>1</sup> J. Morgenroth, Deflection of Complement by Means of Hæmolytic Amboceptors. *Centralblatt Bact.* 1904, Vol. 35, No. 4.

<sup>2</sup> Ehrlich and Morgenroth. See page 88.



results. The plurality of amboceptors is further demonstrated by the results of the isolysin experiments published by Ehrlich and Morgenroth,<sup>1</sup> for in these experiments the presence of antibodies acting against the complementophile group of the amboceptor can be excluded. The fact that we have drawn an incorrect conclusion from one single experiment certainly does not justify Bordet in denying the existence of a plurality of antibodies (especially amboceptors) in a given immune serum; the correctness of our view is established by a number of incontestable experiments.

Bordet's arguments concerning deflection of complement by an excess of amboceptor may be answered in the same manner. Even granted that Morgenroth's view<sup>2</sup> is incorrect, namely, that the inhibition of hæmolysis on the addition of an amboceptor-antiamboceptor mixture is due to a deflection of complement, this would not in the least refute the results obtained by Neisser and Wechsberg with bactericidal sera. In these experiments absolutely no antiamboceptor is present; there are merely bacteria, amboceptor, and complement. Despite this, however, there is no bactericidal action when a certain excess of amboceptor is present. The only explanation for this is the one offered by Neisser and Wechsberg,<sup>3</sup> namely, that the complement is deflected from the amboceptor combined with the cells by the free amboceptor. This explanation has also been accepted by Lipstein,<sup>4</sup> who controverted a number of objections which had been made by various authors. Bordet does not even attempt to controvert our explanation, but contents himself by saying: "Pour nous, la théorie de la déviation du complément par l'ambocepteur est une légende." Needless to say this will have little effect on our view.

It is thus seen that Bordet's recent experiments have furnished additional important confirmation of the amboceptor theory. Analysis of the antiamboceptor action clearly demonstrates the fact that the amboceptor possesses other affinities besides those of the cytophile group; and the circumstance that the occupation of these groups bars the action of the complement shows that they are complementophile in character. Bordet's attack on the receptor theory has thus

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<sup>1</sup> Ehrlich and Morgenroth, Third Communication. See page 23.

<sup>2</sup> J. Morgenroth, l. c.

<sup>3</sup> M. Neisser and Wechsberg. See page 120.

<sup>4</sup> A. Lipstein, *Centralblatt für Bacteriologie*, 1902, Vol. 31, No. 10; see also page 132 of this volume.

failed utterly; his experiments, on the contrary, are to be welcomed as supplementing the arguments supporting the amboceptor theory.<sup>1</sup>

<sup>1</sup> The mistake contained in our previous conception of antiamboceptors, that they were antibodies directed against the cytophile group, is essentially one regarding the situation of the point of attack. In this connection we may look upon certain chemical substitutions as furnishing ready comparison; for example, the different substances resulting when the benzole nucleus is substituted in the ortho, meta, or para positions. Considering how difficult these problems are, it is not surprising that a statement concerning localization will now and then be made which subsequent deeper study shows must be corrected. Even so high an authority as Kekulé once erred in defining a compound, and yet this did not in the least affect his fruitful hypothesis. In our case after the way had been cleared by the demonstration of the "blocking of complements" (the nature of which corresponds to an antiamboceptor action), and by the studies of Pfeiffer and Friedberger, it was an easy matter to arrive at a correct interpretation and transfer the site of the antiamboceptor's action to the complementophile group. It is at once clear that this merely fulfills an old postulate of the side-chain theory. It would therefore be interesting to see how Bordet could explain the facts according to his sensitization theory, and to have him show how the sensitizers, which he believes do not combine with the complement, excite the production of substances whose constitution is just what would be demanded of immunization products of "complementophile groups."

## XLI. A GENERAL REVIEW OF THE RECENT WORK IN IMMUNITY.<sup>1</sup>

By PAUL EHRLICH.

Two years have elapsed since the appearance of my "Collected Studies in Immunity" in Germany, and now that the book is about to appear on the other side of the ocean it is a pleasure for me to review briefly the progress made in that time, naturally without pretending to give a complete résumé of the literature.

I may at once say, however, that very little really new has been added to the views formulated by myself and my collaborators, and that the stereochemical conception of the immunity reaction, despite numerous attacks, has proven itself able to dominate every phase of the subject.

The arithmetical view of the toxin-antitoxin reactions and their analogues, which was introduced chiefly by Arrhenius and Madsen, has invariably shown itself to be untenable. It has led to a numerical science which is far removed from the principles of biological investigations and from the experimental results underlying these. On the other hand, so able an authority as Nernst at once recognized that the laws of chemical equilibrium are not applicable to mixtures of toxin and antitoxin. In addition to this von Dungern, Morgenroth, and Sachs have collected considerable new experimental evidence which demonstrates absolutely that the toxin-antitoxin combination gradually becomes firm, although it may in some instances be quite loose in the first stage. The complex constitution of the poison solutions has thus been conclusively demonstrated; and I may also remind the reader that there can also no longer be any question as to the independent existence of toxons in diphtheria poison, for van Calcar has succeeded in a direct separation of these bodies.<sup>2</sup>

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<sup>1</sup> This chapter is written expressly for this American edition.

<sup>2</sup> van Calcar effected this by means of an ingenious dialyzing procedure (Berlin. klin. Wochenschr. No. 39, 1904). Certain objections raised by Römer

In view of the extraordinary success which physical chemistry has scored, it is readily understood how tempting it was for so eminent a representative of this science as Arrhenius to apply its principles to the new field of immunity. I have always emphasized the chemical nature of the reaction, and am glad therefore that the attempt to apply these principles has been made. It has demonstrated anew that the phenomena of animate nature represent merely the resultants of infinitely complex and variable actions, and that they differ herein from the exact sciences, whose problems can be treated mathematically. The formulas devised by Arrhenius and Madsen for the reaction of toxins and antitoxins explain absolutely nothing. Even in particularly favorable cases they can merely represent certain experimental results in the form of interpolation formulas. Neither do I believe that the phenomena observed in toxins and antitoxins bear any relation to the processes of colloid chemistry. The attempt which has been made to interpret the immunity reaction from the standpoint of colloid chemistry, a subject itself more or less obscure, is based on purely external analogies. I see absolutely no advantage in such a method, and I have grave fears that it will result in checking further progress along this line. Structural chemistry, on the other hand, has not only served to explain all the phenomena in immunity studies, but has also proved a valuable guide in indicating the lines along which further progress might be made. The limitations of colloid chemistry have already manifested themselves, and enthusiastic advocates of this science have been compelled to assume the existence of specific atomic groupings in accordance with my views. I therefore see no reason for abandoning the views expressed in my receptor theory, a theory in complete accord with the principles of synthetic chemistry. My decision finds additional support in the fact that the studies in immunity are constantly bringing to light new observations best harmonized with the views of structural chemistry. Thus I may remind the reader that Morgenroth has recently very cleverly proved the postulate that the components of the neutral toxin-antitoxin combination can be restored. This author succeeded in completely recovering the two components of a neutral mixture of cobra venom

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(Berl. klin. Wochenschr. No. 8, 1905) have been effectually answered by van Calcar by means of some additional experiments, and by the demonstration that the membranes employed by Römer were unsuitable (Berl. klin. Woch. No. 43, 1905).

and antitoxin by means of an ingenious method. But even here we are not dealing with a reversible reaction, for it requires certain manipulations to disrupt the neutral combination; thus, in the case of cobra venom, the addition of hydrochloric acid is necessary. The neutral cobra-venom-antitoxin combination therefore behaves like a glucoside, which in itself is entirely stable, but is split up by the addition of hydrochloric acid.

Besides this, the interesting investigations recently published by Obermayer and Pick,<sup>1</sup> on the production of immune precipitins by means of chemically altered albuminous bodies, are of particular significance in connection with the chemical conception of the immunity reaction. These authors succeeded, by iodizing, nitrifying, and diazotizing animal albuminous bodies, in so changing them that, when introduced into the organism of the same or of different species, they excited the production of precipitins which lacked specificity. These precipitins, however, were strictly specific for their respective iodized albumins, xanthoproteids, or diazo-albumins, no matter from what animal species the albumins were derived.

We see, therefore, that the introduction of a certain chemical group into the albumin molecule completely alters the latter's power to excite the production of antibodies. This certainly corresponds entirely to the view that the production of antibodies is dependent on the chemical constitution of the exciting agent, a view which finds expression in my receptor theory.

The heuristic value of the receptor idea, the idea which underlies my side-chain theory, can best be appreciated by studying the development of our knowledge concerning the cytotoxins of blood serum. As a prototype of these substances the hæmolysins occupy a prominent place in this volume. The view that the hæmolytic immune bodies are amboceptors has been proven to be correct in every case, thus conclusively showing that Bordet's sensitization theory is untenable. To begin, the observations of M. Neisser and Wechsberg, that the action of bactericidal sera depends not only on the absolute, but on the relative concentration of amboceptor and complement, presented conditions which could not be harmonized with Bordet's views. On the other hand, they were readily explained in accordance with the side-chain theory by assuming that the complement was deflected by an excess of amboceptor. But even if this expla-

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<sup>1</sup> Centralbl. f. Physiologie, Vol. XIX, No. 23.

nation is not the correct one, as Gay has recently stated, it would in no way affect the soundness of the amboceptor theory. The existence of amboceptors is confirmed by so many experimental considerations that it is no longer a postulate of the theory, but is practically the direct expression of observed phenomena. The term amboceptor, of course, is used merely to express the two-sided affinity, to the cell on the one hand and to the complement on the other. The affinity of the amboceptor to the cell was demonstrated by the combining experiments published by Morgenroth and myself; and the direct union of amboceptor and complement is confirmed by a host of decisive observations. Of these, it will suffice to mention the test-tube demonstration of complementoids which occupy the complementophile groups of the amboceptor. This demonstration has since been effected in other ways (Fuhrmann, Muir, Browning, and Gay), so that the existence of complementoids is no longer evidenced merely by the possibility of producing anticomplements by means of inactivated serum, but is demonstrated primarily by the unmistakable interference of the complementoids in hæmolytic test-tube experiments. It is not necessary that complementoids should always exert an inhibiting action on hæmolysis; for it is obvious that changes in affinity may occur in consequence of external influences, physical, chemical, or chronological in nature. I believe that changes in affinity, either positively or negatively, are of the highest importance in correctly understanding the course of immunity reactions, although I do not deny the influence of certain catalytic factors on these processes (von Behring, Morgenroth, Otto, and Sachs). However, no general rule can be laid down. Experiments are constantly bringing forth surprises, but by diligent empiricism it is usually possible to bring the many different observations into harmony with a single point of view.

The original assumption, that amboceptor and complement (at least in the case of hæmolysins) exist free side by side, and that the complement does not take part in the reaction until the amboceptor has been bound by the cell (owing to an increase in the affinity of the complementophile group),—this assumption has not proven tenable in every case. In addition to the case described in a previous chapter by Sachs and myself, we now know of a number of combinations, discovered by Sachs, in which the amboceptor alone does not unite with the receptor of red blood-cells, or does so to only a slight degree. By combining with the complement, the amboceptor

has the affinity of its cytophile group increased, so that now it is able to unite with the cells. Thus far, such observations have been made only on normal amboceptors; and this fact explains why the numerous attempts of various authors to separate normal hæmolysins, by means of absorption at low temperatures, have failed.<sup>1</sup> The amboceptors obtained by immunization, on the other hand, regularly possess a high affinity for the cell-receptor. This is easily understood if we consider their mode of origin, for we may perhaps see in this a selection of the groups with the highest affinity. Certainly in this case the exception proves the rule; for the mere fact, that in some instances the amboceptor does not unite with the cell until it has first combined with the complement, at once shows that we cannot be dealing with a sensitization. On the contrary, this shows that the amboceptor is an interbody in the strict sense of the word. These conditions have been most clearly brought out by the experiments of Preston Kyes on cobra venom. The researches of Flexner and Noguchi, as we all know, showed that cobra venom by itself is no hæmolysin, but plays the rôle of amboceptor in hæmolysis. The most important of the activators is the one discovered by Kyes, namely, lecithin. The relation between snake venom and lecithin is really the same as that between amboceptor and complement; but the former possess one great advantage for chemical analysis,—they are both stable substances, and thus contrast strongly with the highly susceptible substances found in blood serum. Hence what was impossible in the case of the latter could readily be effected with cobra venom. Kyes, it will be remembered, has demonstrated, *ad oculos*, the direct union of cobra amboceptor and lecithin complement, and has furthermore succeeded in isolating the resulting combination, the cobra-lecithid, in pure form.<sup>2</sup>

Thus, for the first time, the conclusion was reached chemically

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<sup>1</sup> In this connection I should also like to mention the interesting atypical behavior discovered by Donath and Landsteiner in the amboceptor reaction. These authors observed hæmolytic autoamboceptors in the serum of a patient suffering from paroxysmal hæmoglobinuria. These autoamboceptors, however, only united with the bloods at low temperature.

<sup>2</sup> Kyes has recently continued his studies at my laboratory, and has demonstrated the important fact that in this formation of cobra-lecithid there is a true chemical synthesis. The course of this synthesis is such that a fatty acid radical is split off from the lecithin molecule, whereupon the residual combination, which corresponds to a monostearyllecithin, unites with the cobra ambo-

which, as a result of biological experiences, I had always looked forward to.

The correctness of the amboceptor theory formulated by Morgenroth and myself is confirmed by another important link in the chain of evidence. As far back as 1900, in the Croonian lecture, I stated that, according to the amboceptor theory, three antilytic antibodies were possible. In addition to the substances which act as anticomplements, we could conceive of antiamboceptors of two different kinds. One of these inhibits the action of the amboceptor by preventing the union of amboceptor and cell, the other by occupying the complementophile groups. So far as the confirmation of the amboceptor theory is concerned, it is evident that the demonstration of antiamboceptors directed against the complementophile group is by far the most important; for, owing to the mode of origin, the development of cytophile groups of the amboceptor as reaction products of the specific counter-group (the cell-receptor) is self-evident. It was therefore particularly gratifying when I found that Bordet had recently furnished the demonstration that the antiamboceptor developed with an immune, or with a normal serum, is usually directed against the complementophile group. This discovery very prettily demonstrates that the mechanism of hæmolysin action proceeds according to the amboceptor theory. The error contained in our earlier conception, that anti-immune bodies were usually antibodies directed against the cytophile group, is practically only an error in the localization of the point of attack. This must now be corrected by regarding the complementophile group as the point attacked by the antiamboceptor.

We know that it is possible to produce antiamboceptors by immunizing with normal serum, and Pfeiffer and Friedberger have shown that the action of the antiamboceptor serum extends to all the amboceptors of the animal species whose serum was used for immunization. These facts are only apparently a contradiction of the specificity of amboceptors, for the specificity of the amboceptors applies only to the cytophile group. On the other hand, we must assume that all the amboceptors of the same animal species are at least partly similar in structure so far as the complementophile

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ceptor. This of course destroys the foundations of Noguchi's calculations, which are based on the assumption that the reaction is reversible; it also disposes of certain statements made by Bredig.



apparatus is concerned. In a way, therefore, the amboceptor bears the stamp of the animal species from which it is derived. In this connection I have already expressed my views in the article entitled "The Mechanism of the Amboceptor Action and its Teleological Significance" (Koch Festschrift, 1903): "In general, the specific amboceptors possess a uniform structure in their complementophile portions, whereas they differ to a high degree in their cytophile groups, whose physiological function is the absorption of foodstuffs."

The studies of antiamboceptors have demonstrated that this conception is correct. We see, therefore, that the specificity of the complementophile group of the amboceptor, a specificity based on the animal species, at once leads to a difference in the amboceptors obtained from different species by means of the same immunizing material. In our Sixth Communication on Hæmolysins, Morgenroth and I published certain experiments showing that by means of an antiamboceptor we had been able to demonstrate the diversity of the amboceptors produced in different animal species by injections of ox-blood. This statement still holds good, and its direct consequence demands that in the practical application of bactericidal sera, we should mix immune sera derived from different animals.

In view of Bordet's observation, however, we shall have to revise our interpretation in so far as the site of this differentiation is concerned; the difference is in the complementophile group instead of in the cytophile group. On the other hand, we must abandon the differentiation of partial amboceptors in one and the same serum by means of antiamboceptors, a differentiation which we proposed in the study on hæmolysins. It must not be thought, however, that the pluralistic conception of the amboceptor apparatus is thereby overthrown. This conception is supported by so many arguments of a different kind that the existence of partial amboceptors can be classed as one of the demonstrated facts in immunity. I may remind the reader that by means of mutual elective absorption it is possible to differentiate the strictly specific portion of an immune serum from the non-specific components which give rise to the group reactions. By this means the presence of different amboceptor fractions could be demonstrated in the same immune serum. The observations made by Morgenroth and myself on isolysins also speak strongly in favor of a multiplicity of amboceptors. In these the possible presence of antibodies acting on the complementophile portion of the amboceptor is absolutely excluded. Finally, if we glance at the con-

ditions existing among bacteria, we find the so-called group reactions showing that the receptor apparatus and the antisera possess a highly multiple constitution. This fact, as is well known, has here been of great practical value. We see, therefore, that the plurality of the amboceptors, so far as the cytophile group is concerned, is an assured fact; the differentiation by means of antiamboceptors directed against the cytophile group can therefore very well be foregone. The production of antiamboceptors against the cytophile group seems to encounter particular difficulties, for the complementophile group always finds the corresponding counter group in the organism more readily than does the cytophile group, and therefore is alone bound by the tissue receptors. It is possible that in order to successfully immunize with cytophile groups, it will be necessary to isolate these groups. The latter might be accomplished by neutralizing the complementophile group with the corresponding antibody, or by destroying this group (=cytophilic amboceptoids).

In any event these studies confirm the correctness of the amboceptor theory, i.e., that there is a direct combination of amboceptor and complement. To repeat, therefore, the specificity of the amboceptors applies:

- (1) To the receptor employed in immunization, and this manifests itself in the configuration of the haptophore group; and

- (2) To the animal species from which the amboceptor is derived. The latter kind of specificity shows itself in the structure of the complementophile apparatus, which, as we know, consists of a large number of individual complementophile groups. To this plurality of the complementophile groups there corresponds a plurality of complements as can hardly longer be questioned. So far as the constitution of the complement is concerned, the fact that it is made up of a haptophore and a toxophore group is sufficiently proven by test-tube experiments. The indirect method first employed for the demonstration of the haptophore group, namely, by the production of anticomplements, can therefore be dispensed with.

However, I am convinced that just as normal body-fluids so often contain anticomplements, it will also be found possible to produce these by immunization. But as Moreschi has well pointed out, the experiments by which it was sought to demonstrate the production of anticomplements are not absolutely conclusive. Recent studies by Gengou, Moreschi, and Gay have shown that in the immunization with serum, antibodies directed against the albuminous constituents

are formed which, by uniting with the corresponding albuminous bodies, possess the property of exerting anticomplementary effects. In this case, therefore, the anticomplement action is brought about by the interaction of two components, one present in the serum of the immunized animal and the other in the serum of that animal species whose serum was used for immunization (Moreschi). It is clear, of course, that here the dissolved albuminous substances, not the complements, were the antigens. This being the case, the demonstration of anticomplements produced by immunization becomes extremely difficult, and it must be left for future investigations to see whether it is at all possible to differentiate these substances from those antibodies against albuminous substances which exert an anticomplement action. So far as the mechanism of the described anticomplement action is concerned, I do not think that the observations of Moreschi and Gay, that absorption of complement is associated with precipitation, necessarily mean that precipitation and anticomplement have any causal relationship. In fact it seems reasonable to assume, in accordance with Gengou's first explanations, that the property of binding the complements is exercised by the albuminous bodies sensitized with the specific amboceptor. We would have to conceive this somewhat in this fashion, that just as when immunizing with cells, agglutinins and amboceptors are formed, so also when immunizing with dissolved albuminous bodies two kinds of antibodies are formed, precipitins and amboceptors. If the latter, however, are really amboceptors in the sense of Ehrlich and Morgenroth, we must demand that they will have the same properties which we have always ascribed to the amboceptor type. As a matter of fact, the experiment shows that this is the case. These albumin amboceptors also, in order to react with the complements, must have the affinity of their complementophile apparatus raised, only in the present case this is effected by the combination of the amboceptor with the susceptible body, the albumin. We see, therefore, that this anticomplementary action corresponds to the deflection of complement through an excess of immune body, first described by M. Neisser and Wechsberg. Only in this case the deflecting amboceptor is of a different kind, and needs first to react with the corresponding receptor.

Through the researches of Wassermann and Schütze and of Uhlenhuth, one class of antibodies against dissolved albumins, namely, the precipitins, has been used, as is well known to differentiate albuminous bodies of various origin. These have thus come to be successfully

employed in the forensic demonstration of the origin of blood-stains. The same thing, of course, was possible in the case of the albumin amboceptors.

This fact has recently been taken advantage of by M. Neisser and Sachs,<sup>1</sup> who have devised a procedure by which, by deflecting hæmolytic complements by means of albuminous bodies loaded with amboceptor, they diagnosticate human blood, etc. The study of immunity thus furnishes two biological methods for deciding a point of vital importance in forensic medicine, namely, the origin of blood-stains. Considering the extreme importance of tests of this kind, I am convinced that hereafter it will be well to use this method in addition to the well-tried Uhlenhuth-Wassermann reaction.

This brief résumé, I believe, covers the chief points which have recently come up for discussion, and it is indeed gratifying to me that all the vital questions have been decided in favor of my views. I have gladly applied the results obtained in experimental investigations to an extension of my views, for it is obvious, considering the rudimentary character of a new science, that any successful prosecution of the work will also extend the theoretical conceptions. If then, in spite of this, all the facts brought to light fit naturally into the views formulated by me, I regard this as additional evidence that these views are not so much a theory as a necessary abstraction of the observed facts, an abstraction which is necessary not only in order to obtain a clear and harmonious conception of all the various observations, but also to furnish a scientific basis for a further successful development of the subject.

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<sup>1</sup> Berlin. klin. Wochenschr. No. 44, 1905, and No. 3, 1906.

## XLII. THE MULTIPLICITY OF ANTIBODIES OCCURRING IN NORMAL SERUM.<sup>1</sup>

By Dr. MAX NEISSER, Member of the Institute.

FOLLOWING the fundamental researches made by Flügge and Buchner and their pupils on the bactericidal power of normal blood, we have come to recognize a large number of properties possessed by normal serum. According to our present knowledge we must regard these properties as due to the presence of antibodies in the broadest sense.

Thus far the only theory which has satisfactorily accounted for the origin of these antibodies, from a physiological standpoint and without invoking the aid of teleological "protective substances," is Ehrlich's Side-chain Theory. According to this the cells of the organism produce substances, side-chains, whose physiological function, so long as they are part of the cell, is to lay hold of certain foodstuffs. Side-chains thus anchored are replaced by the cell, and when this regeneration is excessive, the surplus side chains are thrust off into the blood. As a result of this, the blood serum contains a large number of different side-chains. For example, one variety of these side-chains may happen to have an affinity for a particular toxin; it will be found possible, by carefully injecting this toxin, to increase the regeneration and thrusting-off to an extraordinary degree, and thus an immunity is produced against that toxin. From this standpoint, then, immunity is regarded as merely a quantitative increase in the exercise of a normal function.

This view has important bearings on our conception of the antibodies occurring in normal serum. It is apparent that the diversity of the antibodies which can be produced artificially, is entirely analogous to the variety of antibodies present normally.

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<sup>1</sup> Reprinted from *Deutsche med. Wochenschr.*, No. 49, 1900.

This plurality of normal antibodies, advocated by Ehrlich in a number of papers (8, 9, 11), is strongly combated by Bordet by Buchner, who adhere to a unitarian conception. These authors (5) agree that hæmolysins and bacteriolysins are made up of two parts; while they admit that the "interbody" is different, they insist that only a single ferment-like substance, the "alexin," is involved in the lysis of all the various species of blood or bacterial cells.

Kraus (12) goes still further. He found that rabbit erythrocytes could be protected by normal horse serum against several different blood poisons, and concluded "that any given hæmolytic poison acting on rabbit blood, can be paralyzed in its action by means of normal horse serum."

In view of the theoretical importance of this subject, we have thought it advisable to study the question of the unity or plurality of normal antibodies. In doing this we have studied experiments already reported and have supplemented these with some observations of our own.

So far as the hæmolysins are concerned, it has long been known that many sera have the power to dissolve the blood-cells of a number of other species. It is only recently, however, that we have learned how easy it is to produce artificial hæmolysins by immunization. The specificity of these artificial hæmolysins was first demonstrated by Bordet (2), but it was not until Ehrlich and Morgenroth devised elective absorption tests (10) that the subject became clear. This procedure is based on Ehrlich's conception of a chemical union of erythrocytes and hæmolysin; it consists in saturating a serum which contains several hæmolysins, with erythrocytes of one of the species, under conditions which prevent the solution of these cells. Under these circumstances the erythrocytes combine with their specific hæmolysin, and abstract it from the fluid. On centrifuging, it is found that the fluid contains only the remaining hæmolysins, and these have not diminished in amount. By means of this procedure, Ehrlich and Morgenroth (11) demonstrated the existence of several distinct specific hæmolysins in a *normal* serum. They showed that a normal goat serum which dissolved the blood-cells of guinea-pigs and rabbits, could be freed from one of these hæmolysins by treatment with the corresponding blood-cells, the other hæmolysins remaining unaffected. It is to be noted, however, that the hæmolysins consist

of two parts, which Ehrlich terms interbody and complement respectively. The interbody combines with the erythrocyte on the one hand, and with the active dissolving agent, the complement, on the other. The experiments just described, therefore, demonstrated merely the plurality of the interbodies, and shed no light on the unity or plurality of the complements. In fact it was easily conceivable that a single complement (the alexin of Buchner and Bordet) fitted to both interbodies and effected the solution of both species of erythrocytes. Ehrlich and Morgenroth, however, were able to demonstrate that the complements concerned were different. They filtered a serum through Pukall filters, and so effected a separation of the two, one of the complements passing through completely, while all but traces of the other were held back. It was thus shown that the hæmolytic "power" of the normal goat serum against rabbit and guinea-pig blood was due to at least four distinct substances existing independently in the serum side by side.

Nuttall<sup>(17)</sup> was able to show that normal rabbit blood was bactericidal for *B. anthrax*, *B. subtilis*, and *Bact. megatherium*; Nissen<sup>(18)</sup> demonstrated the bactericidal power of rabbit blood on cholera and typhoid bacilli, and on *coccus aquatilis*, and Buchner<sup>(4)</sup> found that cell-free blood serum of rabbits acted on anthrax, erysipelas of swine, typhoid bacilli, cholera, etc. There is considerable variation in the action of the serum, on different bacteria. Thus Nuttall found that rabbit blood acted on anthrax bacilli, but not on staphylococcus aureus. On the other hand, different sera behave differently on the same species of bacterium. Thus Buchner found ox and horse serum without effect on typhoid bacilli. The question again arises, whether the bactericidal action of normal sera is due to a single substance or to different substances.

Experiments to decide this question were made by Nissen<sup>(18)</sup>, although it must be admitted that they were not entirely conclusive. He injected a rabbit intravenously with large quantities of the *coccus aquatilis* and observed that the blood obtained immediately after had lost its bactericidal power for this coccus, while the bactericidal power for cholera and typhoid bacilli remained unchanged.

Extensive investigations concerning this point were then made by Bail<sup>(1)</sup> who employed the absorption test. He found on

on adding dead staphylococci in not too large quantity to rabbit serum, that the clear fluid separated by the centrifuge was still bactericidal for typhoid bacilli, but not for staphylococci. The test also succeeded when done *vice versa*, and with staphylococci and cholera, as well as with typhoid and cholera bacilli.

By means of the absorption test I was able to demonstrate that the bactericidal substances of normal rabbit serum were independent of the hæmolytic substances. Thus, on adding anthrax bacilli to normal rabbit serum, and then centrifuging, it was possible to remove the bactericidal power against anthrax without in any way impairing the hæmolytic power of the serum for goat and sheep blood-cells.

From what has been said it will be seen that the bactericidal action which normal rabbit serum exerts on different species of bacteria is found, by experiment, to be due to several distinct substances in no way dependent on one another.

In the case of another class of antibodies, the agglutinins, recent investigations have shown that they too may exist preformed in normal serum. Here again the question arose whether but a single substance was concerned, or whether there were many different substances.

The first experiments in this direction were made by Bordet (3), who studied normal horse serum. This has the power to agglutinate cholera and typhoid bacilli. By means of the absorption technique of Ehrlich and Morgenroth, Bordet found that after centrifuging serum which had been saturated with one of the organisms, the agglutinating power for that organism would have been lost, while that for the other organism would still be present, and *vice versa*. Subsequently Malkoff (14) reported similar results with red blood-cells. He found that normal goat serum agglutinated (without dissolving) the erythrocytes of the rabbit, pigeon, and man, while the erythrocytes of other animals were but little or not at all agglutinated. Furthermore, it was found that there was considerable individual fluctuation in the serum of different goats. Working with the goat serum, which agglutinated the three bloods just mentioned, he found that by adding, for example, pigeon erythrocytes and then centrifuging, the centrifuged serum would have lost its agglutinating power for pigeon erythrocytes, but was still able to agglutinate the other two species of blood-cells. The experiment succeeded in all possible com-



binations, so that even when two species of blood-cells were added at once, the agglutinating power for these could be reduced to nil while the power for the remaining species of blood was unimpaired. We see, therefore, that the results are entirely similar to those obtained with the hæmolysins and bacteriolysins; the agglutinating power of normal serum on different species of cells is due to separate and distinct substances contained in the serum.

In addition to the foregoing we may also consider for a moment those antibodies which act, not on bacteria or blood-cells, but on ferments and toxins, in other words, the antitoxins and anti-ferments. These bodies are not known directly, but only indirectly by their neutralizing effect; we know little about their occurrence in normal serum. Landsteiner (13), citing also the older literature, found antitryptic substances in normal rabbit, guinea-pig, and ox serum. Morgenroth (15) found antibodies against rennin and against cynarase in the serum of normal goats and horses. By specific immunization this investigator was able to show that rennin and cynarase were two distinct ferments, and that the antirennin of normal serum was distinct from the normal anticynarase. Morgenroth found that the relative amounts of the two antibodies differed in two horse sera which he investigated.

The existence of normal antitoxins has also been reported. Meade Bolton, and later Cobbett (6) found that a considerable proportion of normal horses had diphtheria antitoxin in their serum, and that the amount of this was very variable. Wassermann (18) found that not a few normal human individuals had diphtheria antitoxin in their blood. Ehrlich (7) encountered a normal horse serum which contained an antibody against tetanolysin, and Krauss (12) found normal horse serum effective against a number of hæmolysins. In a paper which Dr. Wechsberg and I hope soon to publish, it will be shown that we have constantly found, in normal human serum, an antibody against staphylotoxin.

In view of the fact that horse serum protects rabbit erythrocytes against tetanolysin, staphylolysin, and other hæmolysins, Krauss concludes that the protective action is due to a single substance in horse serum, and then concludes further that these hæmolysins differ only quantitatively and not qualitatively. A few exact quantitative experiments would have convinced Krauss that this assumption of the non-specificity of hæmolysins is absolutely incorrect. It can be shown that an antistaphylolysin, artificially

produced by immunizing rabbits, protects only against staphylolysin, and not against tetanolysin. This is well shown in the paper about to be published by us. So also it can be shown that a tetanus antitoxin derived from a horse has a marked protective action against tetanolysin, whereas the protective action against staphylolysin is no greater than that of normal horse serum. Finally, it can be shown that normal horse serum usually protects against tetanolysin and staphylolysin, but not against the hæmolysin of normal goat serum. The last-named, it will be remembered, acts on rabbit blood-cells. These hæmolytic poisons, therefore, differ qualitatively from one another.

We see, then, that the antibody present in normal horse serum does not protect rabbit erythrocytes against all blood poisons, for it is not able to prevent the solvent action of normal goat serum. Furthermore, it will be seen from the following experiment that the protective action against a number of different blood poisons is not due to a single substance. The blood poisons employed were tetanolysin and staphylolysin, and the serum of four normal horses was tested against these quantitatively. To begin, it was necessary to determine the complete solvent dose of tetanolysin and of staphylolysin for one drop of rabbit blood. Then the amount of horse serum which sufficed to completely neutralize (inhibit) this dose was determined. The following is an abbreviated protocol of such an experiment.

The complete solvent dose of the staphylolysin employed (14-day filtered bouillon culture of *staphylococcus pyogenes aureus*) was 0.05 cc. for one drop of rabbit blood. The solvent dose of tetanolysin was 0.25 cc.

TABLE I.

	No. of cc. which entirely Neutralizes the Effect of a Complete Solvent Dose of Staphylolysin.	No. of cc. which Entirely Neutralize the Complete Solvent Dose of Tetanolysin.
Horse serum 1.....	0.025	0.25
Horse serum 2.....	0.075	0.05
Horse serum 3.....	0.025	more than 1
Horse serum 4.....	0.25	0.25

That is to say, the number of doses of antibody contained in each cubic centimeter was

	Antistaphylolysin.	Antitetanolyisin.
For horse serum 1.....	40	4
" horse serum 2.....	13.3	20
" horse serum 3.....	40	less than 1
" horse serum 4.....	4	4

Compared to each dose of antitetanolyisin there were in

Horse serum 1.....	10 doses antistaphylolysin
Horse serum 2.....	0.67 "
Horse serum 3.....	more than 40 doses antistaphylolysin
Horse serum 4.....	1

Such a result, however, can be explained only by assuming the existence of two different antibodies.

The point is proved by another experiment. To a given specimen of horse serum whose antitoxic power for staphylolysin is known, enough staphylolysin is added to completely satisfy the antistaphylolysin. When this has been done it will be found that the antitoxic power for tetanolyisin has not been affected.

Thus we see that wherever the bactericidal, hæmolytic, agglutinating, antifermentative, and antitoxic "powers" of normal sera are carefully analyzed, they are found to be due to separate independent substances for each action. By this we do not mean to say that the origin of these substances is necessarily to be ascribed to the action of the elements against which they are found to be directed. On the contrary, for many of these substances, e.g., diphtheria antitoxin in normal horses, it seems likely that certain normal "side-chains" of whose physiological purpose we are still entirely ignorant *happen* to have affinity to a group possessed by some bacterium, ferment, or toxin.

The presence of an antibody in normal serum merely proves that the animal somewhere possesses certain chemical groups, receptors, which happen to have an affinity to the bacterium in question; and that normally there is a moderate overproduction of these receptors with a consequent appearance of thrust-off receptors in the blood.

This thrusting-off, then, is a physiological process which we are able to influence by immunization. As a result of this there is a sudden enormous overproduction of one particular receptor, a kind of pure culture of the receptor grown in the animal. It is obvious, however, that wherever we are able by immunization to cause an excessive thrusting-off of a receptor, there also will it be possible

for such receptors to be thrust off normally. In view of the great diversity of substances which we are able to produce by artificial immunization, it should not surprise us to encounter a great variety of substances in normal serum. When we consider, further, how varied is the behavior of different species and even of different individuals of the same species, we shall at once associate this with the great divergence in the content of normal antibodies in different species and different individuals. As a matter of fact these variations are no greater than the variations in hairiness or in pigmentation.

Further experimental investigations will surely reveal the presence of many more antibodies in normal serum, and it is possible that additional clinico-experimental studies may even give us the key to their physiological function. An insight into their significance in man might open up new ways in diagnosis and therapy.

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### XLIII. THE BINDING OF HÆMOLYTIC AMBOCEPTORS.<sup>1</sup>

By Dr. J. MORGENROTH, Member of the Institute.

It has been established that the hæmolytic amboceptors are bound by the blood-cell receptors for which they have a specific affinity. In earlier papers<sup>2</sup> it was experimentally shown that the amount of amboceptor which can be bound by the blood-cells varies to an extraordinary degree. We call an "amboceptor unit,"<sup>3</sup> the amount of amboceptor which suffices to dissolve a certain quantity of red blood-cells (1 cc. 5% suspension) when plentiful amount of complement is present. Experience has shown that the combining capacity of the blood-cells varies from one to one hundred amboceptor units. On centrifuging the blood-cells after these have bound the amboceptor, and resuspending them in salt solution, it will be found that the amboceptor remains bound unchanged, and is not given off to the fluid in demonstrable quantities at room temperature. It was natural to investigate the firmness of this amboceptor union in suitable cases, namely, cases in which a multiple of the amboceptor unit had been anchored.

By repeated centrifuging and resuspension in salt solution, it is possible to obtain blood-cells laden with amboceptor in a medium entirely free from recognizable traces of amboceptor. A curious phenomenon is observed when fresh blood-cells of the same species are added to such a suspension. After a time some of the amboceptors originally bound to the blood-cells pass over to the new

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<sup>1</sup> Reprinted. from Münchener med. Wochenschrift, No. 2, 1903. A more recent discussion of this subject by the same author will be found in Biochem. Zeitschrift, Vol. XX, 1909.

<sup>2</sup> Ehrlich and Morgenroth, Berliner klin. Wochenschr. No. 10, 1901. This volume, page 71. See also Ehrlich, in Nothnagel's Spez. Pathologie u. Therapie, Vol. VIII.

<sup>3</sup> Morgenroth and Sachs, Berliner klin. Wochenschrift, No. 35, 1902. This volume, p. 254.

blood-cells, so that finally all the blood-cells in the mixture contain an amount of amboceptor, sufficient, when suitable amounts of complement are added, to produce complete solution of the *entire* mixture. This is shown by the following experiments.

To 20 cc. 5% serum-free suspension of ox blood-cells one adds 4.0 cc. inactive serum of a rabbit immunized against ox blood. The complete solvent dose of this immune serum (for 1 cc. 5% suspension) when 0.1 cc. guinea-pig serum is added as complement, is 0.0015 cc. The amount employed in this experiment therefore contained 130 amboceptor units. The mixtures were kept at 38° for one hour, and frequently shaken. The blood-cells were separated by centrifuge, and washed three times with 40 cc. salt solution, and then made up to the volume of the original suspension. The last wash water was free from amboceptor. One cc. of this suspension was mixed with one cc. of a fresh 5% suspension of ox blood-cells, and the mixtures kept for one hour in a water-bath at 40°. On adding 0.2 cc. guinea-pig serum, it was found at the end of fifteen minutes that complete solution of the *entire* quantity of blood had ensued.

This shows that in the course of one hour at 40°, the blood-cells added afterwards had absorbed at least sufficient amboceptor to effect solution. Similar experiments with blood-cells laden with 3, 6, 10, and 60 times the amboceptor unit yielded entirely analogous results. The action takes place even at 0° C., though much more slowly.

The result of these experiments is apparently at variance with earlier statements, that the fluid is free from amboceptors. It is obvious that the amboceptors can only get from one blood-cell to another by way of the fluid medium. The contradiction, however, is explained by assuming that the fluid is *not absolutely free* from amboceptors, but contains such minute traces that they escape detection. When, in the experiment, the blood-cells subsequently added combine with the amboceptors present in the fluid, conditions are produced whereby, in accordance with the law of chemical equilibrium, additional small traces of amboceptor are liberated into the fluid. With the anchoring of this by the fresh blood-cells, the process is repeated, so that the latter bind more and more amboceptor.

In the binding of the amboceptors we are therefore dealing with a reversible process in which the equilibrium is such that

the quantity of amboceptor in solution is usually too minute to be detected. Similar conditions in the solution have recently been described for the hæmolytic substances of certain organ extracts. These substances are only very slightly soluble in salt solution. Nevertheless, when susceptible blood-cells are present at the same time, the substances are anchored by the cells, i.e., abstracted from the solution, while a further minute quantity is given off to the solution. In connection with the experiments made at that time,<sup>1</sup> we called attention to the analogy existing between this phenomenon and certain occurrences in dyeing.

It was necessary, now, to determine how the complete hæmolyisin, i.e., amboceptor plus complement, would behave in an experiment of this kind. The result was highly interesting, for it was found that the ability of the amboceptor to pass from the receptor of one blood corpuscle to that of another existed only so long as the amboceptor had not also combined with complement. On adding *immediately* a suitable amount of complement to mixtures of blood-cells laden with amboceptor and fresh blood-cells, it will be found that only the former are dissolved, i.e., only half of the mixture. Even when the complement is added after 10, 20, or 40 minutes, only part of the blood-cells is dissolved. It is only when the complement is not added until after sixty minutes have elapsed, i.e., after time has been given to permit the passage of sufficient amboceptor, that complete hæmolysis occurs.

Twenty cc. of a 5% suspension of ox blood-cells freed from serum are mixed with 0.048 cc. of the inactive immune serum = 16 amboceptor units. The mixture is kept at 38° and frequently shaken, after which the blood-cells are separated by centrifuging. The blood-cells are washed three times with salt solution until the wash water is entirely free from amboceptor. After making the suspension up to the original volume, 1 cc. doses are mixed with 1 cc. doses of a fresh 5% suspension of ox blood-cells. The mixtures, kept in a water-bath at 40° each, received 0.2 cc. doses of guinea-pig serum at different intervals, namely, at once, and after 10, 20, 40, and 60 minutes. In order to produce the maximum hæmolytic effect, all the tubes were kept in the water-bath for three hours. At the end of that time, half of the blood-cells, corresponding to

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<sup>1</sup> Korschun and Morgenroth, Berliner klin. Wochenschrift, No. 37, 1902. This volume, page 267.

the 1 cc. of sensitized blood-cells, had, of course, dissolved. The degree of solution which the other half had undergone, varied with the length of time after which the complement was added, and is shown in the accompanying table:

	Complement Added.	Degree of Solution.
1	at once	0 to slight
2	after 10 minutes	slight to moderate
3	" 20 "	moderate
4	" 30 "	strong
5	" 60 "	complete

On subsequently adding a further 0.2 cc. guinea-pig serum to tubes 1-4, and placing them in the water-bath, complete solution was produced.

It is not difficult to explain this phenomenon. On adding complement to mixtures of sensitized and fresh blood-cells, the complement is rapidly bound by the anchored amboceptors. We know from earlier experiments that these have an increased affinity for the complement.<sup>1</sup> If the amount of complement is relatively small, while that of the anchored amboceptors is large, it is obvious that only part of the amboceptors will be occupied by complement. The anchored amboceptors which have bound complement are evidently no longer able to let go of their receptor. This fact shows that the anchoring of the complementophile group of the amboceptor produces an increase in the binding power of the cytophile group. The anchored amboceptors which are uncombined with complement, naturally retain their freedom of movement, and are thus enabled to pass over to the freshly added blood-cells. This is demonstrated by the occurrence of hæmolysis on the further addition of complement.

We believe that these experiments constitute an important addition to our knowledge of the relations existing among amboceptor, receptor, and complement. From a well-known experiment made by Bordet,<sup>2</sup> we know that after hæmolysis has begun, amboceptor and complement remain permanently combined. Bordet

<sup>1</sup> Ehrlich and Morgenroth, *Berliner klin. Wochenschr.*, No. 1, 1899. This volume, page 1.

<sup>2</sup> Bordet, *Annales Pasteur*, No. 5, 1901.



determined the quantity of blood-cells which would just be completely dissolved by a hæmolytic serum when the cells were added at once. He then divided the blood-cells into two equal parts, added one part and then the other after the first had been hæmolyzed. The second portion remained undissolved. Bordet incorrectly interpreted this as indicating a physical adsorption of the amboceptor, but, as already indicated,<sup>1</sup> the phenomenon is due to the fact that the blood-cells bind multiples of the amboceptor unit.

Attempts to liberate bacterial agglutinins from their combination with the cells were made some time ago by Hahn and Trommsdorff.<sup>2</sup> These investigators treated agglutinated bacteria with weakly alkaline and weakly acid solutions and actually succeeded in liberating a portion of the bound agglutinin. The agglutinin so liberated was still active. More recently Landsteiner<sup>3</sup> succeeded in liberating the agglutinin from agglutinated blood corpuscles by digestion with physiological salt solution at 50°. This author, moreover, found that even at lower temperatures a certain amount of agglutinin passed into the salt solution used for washing the agglutinated cells, and he therefore concludes, probably correctly, that the combination of cell and agglutinating substance decomposes even at ordinary temperatures, though to a less degree than at higher temperatures.

It is necessary constantly to call attention to the significance of the chemical union of the amboceptors for a correct understanding of the fundamental principles of the immunity reactions. We are here dealing with a chemical binding which is unaccompanied by any toxic action whatever, but which at any time, through the addition of complement, can become manifest by such action. Just this makes it possible to demonstrate the essential distinction between the chemical binding and toxic action, a distinction which finds its expression in the separation of the toxin molecule into a toxophore and a haptophore group. Gruber and Durham<sup>4</sup> were the first to demonstrate the fact that cholera vibrios could remove cholera-immune bodies. Since, however, they identified these

<sup>1</sup> Ehrlich and Morgenroth, loc. cit.

<sup>2</sup> Hahn and Trommsdorff, *Münchener med. Wochenschrift*, No. 13, 1900.

<sup>3</sup> Landsteiner, *Wiener klin. Rundschau*, No. 40, 1902, and *Münch. med. Wochenschrift*, No. 46, 1902.

<sup>4</sup> Gruber, *Wiener klin. Wochenschrift*, No. 12, 1896.

bodies with the agglutinins, they could merely conclude that the agglutinins were used up in the reaction. That a substance is used up as a result of its action, is however, self evident, and constitutes the basis of all dosage. If this were not so we should be able with any poison to produce an endless toxic action, just as theoretically ferment action can go on indefinitely. Although of great importance in itself, all that Gruber demonstrated was the fact that treatment with specifically acting agencies caused the substances to disappear. An insight into the nature of this process, particularly whether it was a destruction or merely a binding, would have required a further systematic analysis, and this was not undertaken. Moreover, just this analysis would have been extremely difficult, because of the views then and perhaps still held by Gruber<sup>1</sup>, namely, that agglutinins and bacteriolysins are identical.

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<sup>1</sup> Gruber, Münchener med. Wochenschrift, No. 48, 1901.

## XLIV. THE JOINT ACTION OF NORMAL AND IMMUNE AMBOCEPTORS IN HÆMOLYSIS.<sup>1</sup>

By Dr. HANS SACHS.

PFEIFFER AND FRIEDBERGER<sup>2</sup> have recently published some very interesting observations concerning the antibacteriolytic action of normal sera. They find, for example, that normal sera which in themselves possess no antilytic power, acquire such power on digesting them with bacteria. Curiously also, the sera thus treated become specifically antilytic, so that a serum treated with cholera vibrios acquires inhibiting properties only against the bacteriolysis of these organisms; a serum treated with typhoid bacilli protects only typhoid bacilli against bacteriolysis.

How is this action to be explained? So far as we know from past experiences, antilytic substances in serum may be either anti-amboceptors or anticomplements. The data contained in the experiments of Pfeiffer and Friedberger leave no room for doubt that anti-amboceptors may be excluded; the authors, however, also declare their disbelief in anticomplements as the cause of the antilytic action, and feel themselves compelled to postulate the existence of new, hitherto unknown substances.

We have carefully studied the experiments reported and believe that two possible explanations present themselves. Thus we may believe that the antilytins in question are anticomplements, which in the native serum, are covered, i.e., hidden, by normal serum constituents. In the digestion with bacteria, these normal constituents are removed (amboceptors). The other possibility is that through the treatment with bacteria the bacterial receptors are liberated in the serum and there functionate as anti-amboceptors. This has already been suggested by Besredka<sup>3</sup>. It is obvious that

<sup>1</sup> Reprinted from *Deutsche med. Wochenschrift*, No. 18, 1905.

<sup>2</sup> Pfeiffer and Friedberger, *Deutsche med. Wochenschr.*, No. 1, 1905.

<sup>3</sup> Besredka, *Bulletin Pasteur*, T. iii, 1905.

the second of these two alternatives would at once explain the specific action of the antilyns. On the other hand, it is difficult to reconcile it with the findings of Pfeiffer and Friedberger, namely, "that it is possible, out of a mixture of inhibiting serum and immune serum, to extract the amboceptor by the subsequent addition of bacteria." While thus compelled to leave open the interpretation of the results reported by Pfeiffer and Friedberger, we should like to report on analogous findings which we encountered with hæmolytic sera in the course of experiments made to check up Pfeiffer and Friedberger's results. Owing to greater ease with which test-tube experiments can be controlled, these experiments proved more susceptible to analysis. The bloods employed were from sheep and pig, and these were hæmolyzed by the corresponding immune sera<sup>1</sup> with guinea pig serum as complement.

Neither combination is inhibited by inactive normal rabbit serum, and yet, as soon as this serum is digested with sheep blood or with pig blood, it is found to have acquired antilytic properties. This inhibition of hæmolysis, moreover, is specific, so that when sheep blood-cells have been used for treating the serum, the inhibition extends only to the hæmolysis of sheep blood, but not to that of pig blood, and when pig blood is used, the inhibition applies only to pig blood hæmolysis.

This is illustrated by the following experiment: To 10 cc. inactive rabbit serum were added the sedimented cells from 10 cc. sheep (or pig) blood; the mixture was kept at 37° C. for one hour, and then centrifuged to separate the serum from the blood-cells. The supernatant fluids thus obtained were added in decreasing amounts to constant quantities (0.1) of active guinea-pig serum, and digested for half an hour; then 1 cc. of a 5% suspension of blood and a suitable amount (1½ amboceptor units) of amboceptor was added. Native rabbit serum was treated in exactly the same manner as the supernatant fluids.

The following table shows the degree of solution noted in the different combinations. The tubes in Column A contained sheep blood plus 0.01 cc. of the corresponding immune serum; the tubes

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<sup>1</sup> The immune serum for the pig blood was obtained by immunizing a rabbit with pig blood; that for sheep blood was obtained by immunizing a rabbit with ox blood, as this was found by Ehrlich and Morgenroth (Berlin. klin. Wochen., 1901, Nos. 21 and 22) to be hæmolytic also for sheep blood.

in Column B, contained pig blood plus 0.015 of the specific amboceptor. The figures in each column denote:

1. Native rabbit serum.
2. Rabbit serum treated with sheep blood.
3. Rabbit serum treated with pig blood.

TABLE I.

Amount of Rabbit Serum. cc.	A			B		
	1	2	3	1	2	3
1.0	complete	0	complete	complete	complete	0
0.5		0				0
0.25		0				0
0.15		ft. trace				0
0.1		slight				faint trace
0.05		slight				slight
0.025		almost complete				moderate
0.015		complete				strong
0.01		"				strong
0.0		"				complete

This table gives a beautiful illustration of the point noted by Pfeiffer and Friedberger, namely, that the rabbit serum, which has no antilytic properties whatever, exerts a specific antilytic action after it has been treated with the corresponding blood-cells. It is a simple matter to show that this antilytic action is not directed against the amboceptors. One need merely mix amboceptor and inhibiting serum, and then digest the blood cells in this mixture. After centrifuging, it will be found that the sedimented blood-cells are readily hæmolyzed on the addition of complement. This, of course, shows that the amboceptor cannot have been affected. Under these circumstances, and in the light of our past experiences, we would ascribe the action to anticomplements, but in doing so we encounter apparently a great difficulty, the specificity of action. But is this specificity really irreconcilable with the assumption of an anticomplement action? It seems to me that no such difficulties exist in our case, and would ask the reader's attention to the following considerations:

It can be shown that the inhibiting effect produced by a serum after digestion with a particular species of blood (in our case with sheep blood), is due essentially to the absorption of normal ambo-

ceptors acting on sheep blood-cells. If one allows the normal amboceptors to participate in the reaction *by themselves*, it will be found that the antilytic effect is not produced.

The demonstration is made as follows: An inhibiting serum, prepared by treating rabbit serum with sheep blood-cells, is mixed with complement (0.1 cc. guinea-pig serum) and allowed to act on sheep blood-cells which have been sensitized in one case with immune serum, in another case with this and normal rabbit serum. The result is shown in the following table.

In Column I the reagent consisted always of 1 cc. 5% sheep blood sensitized with 0.002 cc. immune serum obtained by immunizing a rabbit with ox blood-cells.

. In Column II, the sheep blood was treated in exactly the same manner, and then digested with 0.5 cc. normal rabbit serum, whereupon the blood was freed from serum by centrifugalization.

TABLE II.

Amount of Rabbit Serum Previously Treated with Sheep Blood-cells. cc.	I	II
1.0	0	} complete
0.5	0	
0.25	0	
0.15	faint trace	
0.1	slight	
0.0	complete	

It will be seen from the table, that through the coaction of the normal amboceptors of rabbit serum, the antilytic action disappears, and this at once explains why the inhibiting function should be absent in native serum. The inhibiting antibodies are really present in native rabbit serum from the outset, but they are hidden by the simultaneous action of the normal amboceptors. The experiment further shows that the digestion of serum with blood-cells does not bring about, for example, a tearing off of receptors through the agency of the normal amboceptors. (Such a combination in the serum fluid, moreover, would really act like an anti-complement). Column II shows that the normal amboceptors are really bound by the blood-cells. From the behavior of the various

combinations, we must furthermore conclude that the absence of antilytic action of native serum is only apparent. Haemolysis of the sheep blood-cells by the *immune* serum is inhibited, but in place of this the *normal* amboceptors of rabbit serum come into play and effect haemolysis with the aid of the complement of guinea-pig serum. This, of course, affords a natural explanation for the specificity of the phenomenon. The rabbit serum which was treated with sheep blood-cells has lost the amboceptors for sheep blood, but still contains those for pig blood. Hence it inhibits *only* the haemolysis of sheep blood by immune serum. When the serum is treated with pig blood, the behavior, of course, is just the reverse of this.

This explanation of the specificity harmonizes very well with the view that the inhibiting substances are anticomplements. It is only necessary to assume that the anticomplements act specifically in the sense that under suitable conditions only the immune and not the normal amboceptors are prevented from combining with the complement. It might be assumed, for example, that the activation of normal and immune amboceptors is effected by different complements. It seems simpler, however, to assume that the complementophile group of the normal amboceptors has a greater affinity than that of the immune amboceptor. At first sight this may appear remarkable, but it is not really so. It is true that one can usually regard the immune amboceptors as having the stronger affinity, but this greater affinity applies only to the cytophile group, i.e., the group whose occupation really gave rise to the immunity reaction. So far as the normal amboceptors are concerned, there is another reason for believing that the complementophile apparatus possesses a greater affinity. Anticomplements, as Ehrlich and Morgenroth<sup>1</sup> have already shown, are nothing more than amboceptors which have reached the blood stream. According to this view, artificially produced anticomplements are amboceptors which differ from the amboceptors produced in response to cells injections, only in the fact that their thrusting-off is due to the occupation of their *complementophile* group. Originating in this way, a natural selection of complementophile groups with the greatest affinity, of course, occurs and this subsequently shows itself in the increased affinity for the anticomplements.

<sup>1</sup> Ehrlich and Morgenroth. Fifth Communication on Haemolysins. See page 71 of this volume.

Since experience has shown that normal sera so frequently exert anticomplementary powers, we are compelled to assume that the normal amboceptors, of which, as is well known, large numbers circulate in the blood, generally possess a high affinity to the complement. Thanks to this high affinity they are able to deflect the complement from the amboceptor concerned in the reaction. Naturally, the question whether in a given case the amboceptor is to act as such or as anticomplement, will depend in general on whether it fits the given species of cell or not. In any event, the anticomplementary action as thus conceived corresponds entirely to Neisser and Wechsberg's phenomenon of deflection of complement by an excess of amboceptor,

Returning now to the problem under discussion, we find that this finds a ready explanation along the lines indicated. This will be clear on studying the schematic figure appended.

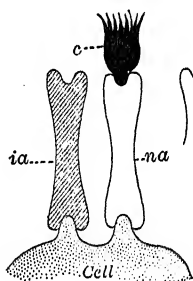


FIG. 1.

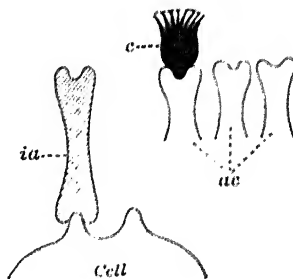


FIG. 2.

IA = immune amboceptor. NA = normal amboceptor. AC = normal amboceptors functioning as anticomplements. C = complement.

In Fig. 1 is represented the action of native rabbit serum on the hæmolysis of sensitized sheep blood-cells by guinea-pig complement. The sheep blood-cells are loaded with the immune amboceptor (IA). The normal amboceptor of rabbit serum fitting to sheep blood-cells (NA) has likewise been anchored by the cell, and has laid hold of the complement.<sup>1</sup>

In Fig. 2 the normal rabbit serum, through digestion with sheep blood-cells, has lost its amboceptor for these cells. Under these

<sup>1</sup> The higher affinity of the normal amboceptors will be still further increased in favor of those bound to the cell, for it is well known that in combining with the cell the complementophile group acquires an increased affinity.



circumstances the free normal amboceptors, which act as anti-complements (AC) come into play and deflect the complement from the immune amboceptor (IA).

It is understood, of course, that in addition to these changes in affinity, some significance must also be attached to the law of mass action. Thus, if a very small quantity of normal amboceptors united to cells is placed beside an enormous number of free anti-complements, it is possible that a deflection of complement may occur. In dealing with native normal sera, such a disproportion is out of the question, for by increasing the quantity of anti-complements there is also an increase in the amboceptors fitting the cell. On the other hand, if the blood-cells have only been slightly sensitized and when then large amounts of the inhibiting serum are employed, a slight antilytic effect may be produced. If due regard is given to the relative amounts of the factors, and the blood-cells are sensitized with the proper proportion of normal serum, no trouble will be experienced in observing the absence of antilytic action against the normal amboceptor. For the sake of completeness the following experiment is appended.

Two series of test tubes are prepared, the first containing 0.1 cc. guinea-pig serum plus decreasing amounts of native rabbit serum, the other containing the same amount of guinea-pig serum plus decreasing amounts of rabbit serum which has previously been absorbed with sheep blood-cells. To each of the tubes is added then 1 cc. 5% sheep blood-cells which have previously been sensitized with 0.5 cc. normal rabbit serum, and separated from the serum by centrifugalization. The result is shown in the following table. The control with immune amboceptor is shown in Column I, Table II.

TABLE III.

Amount of Rabbit Serum cc.	A Native Rabbit Serum	B Rabbit Serum Absorbed with Sheep Blood
1 0	complete	complete
0 5		
0 25		
0 15		
0 1		
0		

A number of observations made during the course of other experiments gives additional support to the view that the inhibiting action is due to anticomplements whose action is hidden, in native serum, by the normal amboceptors. Thus, it was possible to bring about inhibition by absorption, only when the serum employed already contained amboceptors for the blood-cells in question. Where these amboceptors were absent, no change whatever was produced by the absorption, the serum either inhibiting equally well before and after absorption, or not inhibiting at all. Normal rabbit serum, for example, is in no way changed when absorbed with ox blood-cells, because it lacks fitting receptors for these cells. Owing to the fact, however, that it contains anticomplements, rabbit serum even in its native state exerts an antilytic effect on ox-blood hæmolysis, and this action is unaffected by absorption either with ox blood or sheep blood. In this connection the individual variations observed in the behavior of rabbit sera toward sheep blood is most instructive. Thus, I have encountered rabbit sera in which, by chance, the amboceptors for sheep blood were practically absent. These sera, however, even in the native state, possessed an antilytic effect on sheep-blood hæmolysis, and this was unaffected by treatment with sheep blood-cells.

Finally mention should be made of a circumstance which makes it highly probable that the substances in question are anticomplements. We have seen that the inhibiting serum produces its effect when guinea-pig serum is used as complement. On the other hand, no inhibition will be produced if rabbit serum is used as complement. The amboceptors present are complemented with rabbit serum just as well as with guinea-pig serum, and the failure of absorbed rabbit serum to inhibit when rabbit serum is used as complement can be readily understood if we regard the inhibition as due to anticomplements as already set forth, for it is well known that autoanticomplements are uncommon.

It is, of course, impossible for us to say whether the data here reported are applicable to the observations made by Pfeiffer and Friedberger on bacteria. From what has been said it is apparent that the specificity observed by those authors would agree very well with the anticomplement hypothesis. Nor is this hypothesis contradicted by the fact that a certain excess of amboceptor nullifies the paralyzing action of the inhibiting serum. In anticomplement actions the quantitative relations between amboceptor,

nt, and anticomplement are so important that the failure ion when large amounts of amboceptor are employed ascribed to the disproportion between the reacting sub- This phase of the subject has been investigated by Mor- nd Sachs.<sup>1</sup> Finally mention should be made of a fact y Pfeiffer and Friedberger which strongly supports the ument theory. By means of bacterial absorption they in converting normal rabbit, goat, and pigeon serum iting serum, but failed to convert guinea-pig serum. Yet established by experiments in this direction that bacter- es place in the peritoneal cavity of guinea pigs, that, ords, these animals do furnish complement. The negative ained with guinea-pig serum, therefore, may be regarded ng the absence of autoanticomplements, and the exper- rds additional reason for believing that the antagonistic e observed by Pfeiffer and Friedberger are probably uments.

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enroth and Sachs. Berliner klin. Wochenschrift, No. 35, 1902.

## XLV. THE POWER OF NORMAL SERUM TO DEFLECT COMPLEMENT.<sup>1</sup>

By Dr. HANS SACHS, Member of the Institute.

In a previous paper<sup>2</sup> the writer discussed the action of certain substances in normal serum which, according to Pfeiffer and Friedberger,<sup>3</sup> exerted antibacteriolytic effects. A recent study by Gay<sup>4</sup> leads me to take up the subject anew. Pfeiffer and Friedberger had shown that normal sera which by themselves possessed no antibacteriolytic properties, acquired such properties if they were previously digested with bacteria. Moreover the sera obtained by this treatment exert specific antilytic properties, that is to say, a serum digested with cholera vibrios protects only cholera vibrios against bacteriolysis, etc. I thereupon studied the same conditions by means of *haemolytic* test-tube experiments, and was able to confirm the author's findings. Rabbit serum digested with sheep blood-cells exerts antihæmolytic effects directed practically entirely against the hæmolysis of sheep blood. My conception of the mechanism of this action differs from that of Pfeiffer and Friedberger only in that I do not regard the inhibiting substances concerned as new, hitherto unknown bodies. I believe that this inhibiting effect, at least in the case of hæmolysins, is due to amboceptors, acting, as they often do, like anticomplements. That such amboceptors occur in normal serum is well known from numerous observations. At any rate, the views of Pfeiffer and Friedberger and my own probably agree in regarding the inhibiting substances in the serum as *preformed*, their action in native serum

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<sup>1</sup> Reprinted from *Centralblatt f. Bacteriologie*, Vol. XI, 1906.

<sup>2</sup> Sachs. *Deutsche med. Wochenschrift*, No. 18, 1905.

<sup>3</sup> Pfeiffer and Friedberger, *ibid.* No. 1, and also No. 29, 1905.

<sup>4</sup> Gay, *Centralblatt Bacteriologie*, Orig. XXXIX, 1905. See also Bordet-Gay, *Collected Studies*, Wiley & Sons, 1909.

being hidden by the normal amboceptors which are removed by the digestion with blood-cells or bacteria.

Gay believes otherwise. He has repeated my experiments, especially those dealing with hæmolysis, and concludes that my explanation is "certainly incorrect." Gay believes that the cause of the phenomenon described is to be sought in a binding of complement by precipitates. According to him the precipitin is in the sheep-blood immune serum; the precipitable substance is in the rabbit serum digested with sheep blood and comes from traces of serum remaining on the sheep blood after insufficient washing. This explanation, at first sight, seems most reasonable. We know from the researches of Gengou<sup>1</sup> that the combination resulting from the union of serum albumin and a corresponding antiserum has the power to bind complement. Through the recent investigations of Moreschi<sup>2</sup> and of Gay<sup>3</sup> a great deal of interest has been aroused in this property, and M. Neisser and I<sup>4</sup> have reported on experimental studies in which we sought to utilize the complement-binding power of albuminous bodies laden with antiserum in a forensic blood test. For the question here at issue it matters not whether the precipitate as such absorbs the complement, or whether, as we believe, the albuminous bodies are sensitized by specific amboceptors in Gengou's sense, so that they then bind the complement just as do sensitized cells. The main point is that according to Gay's view the inhibition of hæmolysis must be due to the interaction of sheep serum and the immune serum acting on sheep blood.

The experiments made by Gay apparently corroborate his assumption. Thus when the sheep-blood corpuscles used for treating the rabbit serum were washed five successive times with physiological salt solution, he found that the centrifuged rabbit serum no longer produced inhibition, whereas when the serum was treated with sheep blood washed but once, it produced the inhibition which I had described. The difference which I observed in the behavior of normal and immune amboceptors of rabbit serum, so far as the inhibiting action of rabbit serum treated with sheep blood is concerned, Gay believes, is only an apparent one. Normal

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<sup>1</sup> Gengou, *Annales Pasteur*, Tome XVI, 1902.

<sup>2</sup> Moreschi, *Berliner klin. Wochenschrift*, No. 37, 1905.

<sup>3</sup> Gay, *Centralblatt Bacteriologie*, Orig. XXXIX, 1905.

<sup>4</sup> Neisser, M., and Sachs, *Berliner klin. Wochenschrift*, No. 44, 1905.

serum simply does not contain the antibodies (precipitings) acting on the sheep serum, and this is why there is no inhibition. In one experiment, however, I called attention to the fact that the hæmolysis of blood cells sensitized only with immune serum is prevented by the inhibiting serum, whereas blood-cells sensitized with immune serum and then also with normal serum are dissolved under these conditions. In both cases after the amboceptors had been anchored I separated the serum fluid by centrifuging. It so happened that I expressed myself somewhat differently in the second case, and this has led to a misconception. In the second case I said "the blood-cells were digested with serum and then freed from serum fluid by centrifuging"; in the first case I merely said "the reagent used was sheep blood *sensitized* with immune serum." By "sensitized blood," of course, I mean blood-cells which, after treatment with amboceptors, are separated by centrifuge. In fact, in another experiment contained in this study I expressly state "sheep blood+immune serum." However, I am willing to admit that my mode of expression might give rise to doubts. Gay, however, seems to know my experimental technique better than even I myself. He declares simply that I had centrifuged in the second case, i.e., had removed the precipitating portion of the immune serum, and had not done so in the first case. My experiments therefore contained "a grave experimental error." Through his own experiments, Gay believes to have furnished "a complete refutation of Sach's hypothesis."

Gay has made a regrettable mistake. Moreover, in repeating my experiments he has allowed a grave error to creep into his own technique. It really is immaterial, in my experiments, whether the immune serum is centrifuged from the blood-cells or not, since the immune serum I employ has so high an amboceptor content that the quantity used for sensitizing (0.002 cc.) is only about 1-200 of that employed by Gay. According to my experience, this quantity is too small to effect a precipitation or sensitization of the albuminous bodies of the serum. Nevertheless I have made a number of experiments with my immune serum according to the procedure outlined by Gay. I treated rabbit serum with sheep blood washed once, and also with sheep blood washed five times. Both lots of serum so treated proved equally antihæmolytic, whereas native rabbit serum possessed no inhibiting action whatever. This is illustrated by the following protocol.

# POWER OF NORMAL SERUM TO DEFLECT COMPLEMENT. 613

Decreasing amounts of inactive rabbit serum are digested with 0.05 cc. guinea-pig serum. Then 1 cc. 5% sheep blood washed five times plus 0.0015 cc. amboceptor (serum from a rabbit immunized with ox blood) are added. In the following table

- A denotes native rabbit serum;
- B rabbit serum treated with sheep blood washed once;
- C rabbit serum treated with sheep blood washed five times.

Amounts of Rabbit Serum. cc.	Degree of Hæmolysis.		
	A	B	C
1.0	} complete	0	0
0.5		0	0
0.25		0	0
0.15		moderate	moderate
0.1		complete	complete
0		complete	complete

The experiment by which Gay seeks to explain my results is therefore entirely valueless so far as my experiment is concerned. To be sure, Gay believes to have followed my technique exactly, yet in one important point he has not done so. The hæmolytic immune serum with which he worked was markedly weak, the solvent dose being 0.2 cc., while 0.001 cc. of my serum still brought about complete solution. Gay employed 0.4 cc. immune serum, while I used but 0.002 cc., i.e., 1-200 of his dose. It is very well possible that the antibodies which sensitize serum albuminous bodies, the precipitating substances as Gay believes, are present in 0.4 cc. immune serum; in amounts as small as 0.002 cc. they are almost certainly absent, and I never observed any precipitin action with these amounts. It is evident, therefore, that with the large doses of immune serum employed by Gay the presence of slight amounts of sheep serum might well make a difference in the result, while this would be a negligible factor in my experiments.

But how are we to explain the fact that Gay after treating the rabbit serum with sheep blood which had been washed five times was unable to demonstrate the inhibiting action described by me? This again is due to the unfortunate employment of the weak immune serum. Gay has evidently been working with normal amboceptors. It is well known that rabbit serum normally dissolves sheep blood, and the ordinary strength of this hæmolytic power corresponds

entirely to that of Gay's immune serum. The complete solvent dose of normal rabbit serum fluctuates in most instances between 0.25 and 0.1 cc. The solvent dose of Gay's immune serum was 0.2 cc. The quantity employed by Gay, 0.4 cc., probably suffices with any rabbit serum to completely dissolve 1cc. 5% sheep blood on the addition of 0.1 cc. guinea-pig serum. Hence it is not at all impossible that Gay employed a serum which contained no immune amboceptors whatever, and represented merely an albumin anti-serum. If amboceptors were artificially produced, they were certainly present in so small concentration as to be unable to increase the action of the normal amboceptors to any appreciable degree. In my paper, however, I distinctly stated that the inhibiting sera obtained by treatment with blood-cells acted only against hæmolysis produced by *immune* amboceptors, and that this antilytic action was prevented by the action of the normal amboceptors. Just this constituted my explanation for the absence of the inhibiting function in native serum, for I assumed that the inhibiting antibodies were already present in native rabbit serum and were merely hidden by the simultaneous action of the specific normal amboceptors.

Gay's experiments thus constitute an involuntary complete confirmation of my views, and it is to be regretted that Gay has allowed himself to be misled in the interpretation of my experiments. The incorrectness of his views should have struck him from a number of statements in my first paper. If his idea was correct it follows that the inhibiting action should appear in every rabbit serum treated with insufficiently washed sheep or ox blood. I distinctly stated, however, that I could only then demonstrate an inhibiting effect through absorption with blood-cells when the serum under examination from the outset contained amboceptors for the species of blood in question. I said particularly that normal rabbit serum, which contains no amboceptors for ox blood, is in no wise changed by absorption with ox blood, i.e., it neither before nor after treatment with ox blood does it inhibit hæmolysis of sheep blood by immune serum, while on the hæmolysis of ox blood it exerts the same inhibiting power before and after treatment. Finally I called attention to a number of rabbit sera in which, quite exceptionally, the amboceptors for sheep blood were absent. These sera from the outset were antilytic for the hæmolysis of sheep blood, and they remained so in unaltered degree after digestion with sheep blood. After



all this it is absolutely necessary to conclude that the inhibiting substances are already present in native serum, and that their action in this serum is merely disguised by the simultaneous action of the normal amboceptors. If the latter are removed by absorption with blood-cells, the antilytic power of the inhibiting substances becomes manifest. Gay's attempt to refute this conception has thus come to naught, and all because of a circumstance in his technique which Gay himself perhaps not unjustly, would term a "grave experimental error."

## XLVI. THE JOINT ACTION OF SEVERAL AMBOCEPTORS IN HÆMOLYSIS AND THEIR RELATION TO THE COMPLEMENTS.<sup>1</sup>

By Drs. H. SACHS and J. BAUER.

THERE is still no unanimity of opinion concerning the mechanism of the cytotoxic action of blood serum. Most of the authors, to be sure, have accepted the amboceptor theory of Ehrlich and Morgenroth. According to this view, the thermostable components of the serum possess two haptophore groups, one combining with the cell and the other with the complement, the labile component of the serum. Bordet, however, continues most ingeniously to defend an opposing view. In the sensitization theory advocated by this distinguished investigator, the existence of a direct relationship between amboceptor and complement is denied. According to this view, which is based on molecular adhesion, the cell is sensitized by the amboceptor so that it becomes vulnerable to the action of the complement. So far as can be discovered blood cells (which constitute the ordinary material on which to study the mechanism of amboceptor action) do not by themselves react with complement, and it has therefore been impossible to prove the correctness of the sensitization theory experimentally. The theory can only be defended indirectly, by showing that there is no direct relation between amboceptor and complement. Bordet's demonstrations have therefore been limited to pointing out objections in experiments supporting the amboceptor theory. It is not our intention to present all the material bearing on this point. One of us<sup>2</sup> has recently reviewed the subject on the light of our present knowledge. Suffice it to say that the refutation of experiments

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<sup>1</sup> Reprinted from *Arbeiten u. d. kgl. Institut f. experimentelle Therapie zu Frankfurt a. M.* Heft 3, Jena, 1907.

<sup>2</sup> Sachs, *Die Hæmolysine und die cytotoxischen Sera.* Lubarsch-Ostertags *Ergebnisse der Pathologie.* Vol. 11, 1907.

which appeared clearly to indicate the direct union of amboceptor and complement is not at all sufficient to overthrow the amboceptor theory. Attacking our interpretation of a phenomenon which played an important role in proving the existence of direct relations between amboceptor and complement, Bordet and Gay<sup>1</sup> in a recent paper, report an experiment which they believe controverts our view. Going still further, these authors conclude that the amboceptor theory must be abandoned as fallacious. We fail to see the force of this conclusion. For even if the proof adduced by Bordet and Gay in this single instance were accepted as irrefutable, it would only show that the direct demonstration of the amboceptor theory is impossible. The authors have not brought forward a single fact which contradicts the amboceptor theory. If, then, in the following pages we take up at length the observations of Bordet and Gay, it is not because we consider it necessary to renew the old discussion "amboceptor or substance sensibilatrice?" but merely because of the great interest of the observations. Furthermore the interpretation given by the authors is so peculiar that it demands further analysis.

## I

The case discussed by Bordet and Gay deals with a combination previously described by Ehrlich and Sachs,<sup>2</sup> namely hæmolysis of guinea-pig blood through the combined action of inactive ox serum and active horse serum. Ehrlich and Sachs had found that guinea-pig erythrocytes, which can be dissolved by a mixture of inactive ox serum and horse serum, remain intact if they are first treated with inactive ox serum, and then, after removing the ox serum, are digested with horse serum. This showed that the constituent of ox serum has not been bound by the blood cells. It was to be assumed that this constituent represented the amboceptor, and Ehrlich and Sachs therefore rightly concluded that in this case the amboceptor had not been bound by the blood-cells, that it reacted with the cell only after the amboceptor and complement had combined. The same combination was subsequently studied

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<sup>1</sup> Bordet et Gay, *Annales de l'Institut Pasteur*, No. 6, Vol. XX, 1906.

<sup>2</sup> Ehrlich and Sachs, *Berliner klin. Wochenschr.* No. 21, 1902. See also this volume, page 209.

by Klein,<sup>1</sup> who found that horse serum, through digestion with guinea-pig blood, loses its complementing power for the combination "guinea-pig blood inactive ox serum." Finding that the horse serum suffered a loss of its agglutinin at the same time, Klein advanced the view that the complement was destroyed by the process of deglutination. This view was combated by Browning,<sup>2</sup> who showed that the complements of horse serum remain unaffected if the guinea-pig blood-cells are digested with the serum at low temperatures (0° C.), although optimum conditions for the agglutinating action and for the binding of agglutinin are thus presented. Browning believes that the reason for the disappearance of complement through digestion at higher temperatures, lies in the fact that horse serum contains amboceptors for guinea-pig blood, which amboceptors serve to bind the complement only at higher temperatures. That amboceptors for guinea-pig blood exist in horse serum was demonstrated by Morgenroth and Sachs,<sup>3</sup> who show that horse serum plus active guinea-pig serum was able to produce hæmolysis of guinea-pig blood-cells.

These authors demonstrated further that horse serum alone, even in large doses, only rarely dissolved guinea-pig blood-cells. This showed that horse serum usually did not contain the suitable "dominant" complement fitting its own amboceptor for guinea-pig blood. It is well known that an amboceptor which has been anchored to a cell is able to rob an active serum of all its complement functions. Furthermore, according to Ehrlich and Sachs,<sup>4</sup> under certain conditions even "non-dominant" complements may be anchored while "dominant" complements remain in solution. Hence the explanation offered by Browning presented no difficulties. Browning assumed that the horse serum complement, dominant for the ox amboceptor but not dominant for the horse amboceptor, is absorbed by guinea-pig blood through the agency of the serum's own amboceptor. The loss of complement described by Klein was thus readily explained on the basis of the ambo-

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<sup>1</sup> Klein, Wiener klin. Wochenschr., No. 48, 1905.

<sup>2</sup> Browning, Wiener klin. Wochenschr., No. 15, 1906.

<sup>3</sup> Morgenroth and Sachs, Berliner klin. Wochenschr., No. 27, 1902. See also page 233.

<sup>4</sup> Ehrlich and Sachs, Berliner klin. Wochenschr. 1902, Nos. 14 and 15. See also this volume, page 195.

ceptor theory. Browning also showed that a similar effect could be produced with other species of blood which by themselves were unable to rob horse serum of its complement. It was merely necessary to introduce a specific amboceptor. Ox-blood, for example, has no influence on horse serum. Nevertheless, when treated with a specific amboceptor derived from a rabbit, it binds the horse serum complement fitting inactive ox serum, and this binding occurs without the prepared cells being dissolved by the horse serum. According to Browning, therefore, hæmolysis of guinea-pig blood brought about by the combined action of inactive ox serum and active horse serum is to be explained as follows: The affinity possessed by the ox amboceptor for horse complement is greater than that possessed by the free horse amboceptor. Hæmolysis occurs if the ox serum and horse serum are added at the same time. If, however, the horse serum is first digested with guinea-pig blood, the horse amboceptor will unite with the blood-cell. This union leads to an increase in the affinity of the complementophile group and causes the complement to be anchored to the horse amboceptor. The union between complement and amboceptor becomes more and more firm, so that after a time not even the ox amboceptor, which really possesses a higher affinity than the horse amboceptor, is able to disrupt the combination. (See figures 1 and 2 of the accompanying plate.)

It is apparent that Bordet and Gay were unacquainted with the work of Browning. The experiments they report are largely identical with those made by Klein and Browning. The following interesting experiment, however, is entirely new: Ox blood-cells loaded with amboceptor do not dissolve in horse serum, but do dissolve in a mixture of active horse serum plus inactive ox serum. In this case, the authors rightly reason, the ox serum cannot possibly act as an amboceptor, but must represent a constituent necessary for hæmolysis, but identical neither with the amboceptor nor with the complement. Analogously, in the combination guinea-pig blood plus inactive ox serum plus horse serum, the horse serum is believed to act, not as an amboceptor, but as a third component effecting hæmolysis. Bordet and Gay thus assume that amboceptor and complement are present in horse serum but are unable to effect hæmolysis without the presence of the third component present in ox serum. This hypothetical substance they term "*colloide de bœuf*." According to Bordet and Gay, this colloid

has the following properties: It is stable, resisting long standing and heating to 56°. It is bound by the blood-cells only after these have been loaded with amboceptor and complement. When so bound it effects agglutination and hæmolysis. Bordet and Gay thus assume the existence of an entirely new substance in horse serum, and ascribe to it very important properties. The interpretation which these authors give of the phenomenon described by Ehrlich and Sachs is merely an hypothesis entirely lacking in proof. Granted that the rôle of the ox serum in the hæmolysis of sensitized ox blood by means of horse serum cannot be looked upon as an amboceptor action, this by no means justifies the analogous conclusion that in the hæmolysis of guinea-pig blood by inactive ox serum and horse serum the ox serum does not play the part of an amboceptor. It should at least be shown that guinea-pig blood digested with horse serum (whereby, according to the view of Bordet and Gay, amboceptor and complement are bound) is hæmolyzed on the subsequent addition of inactive ox serum. Klein and Browning, however, showed that this was not the case. The latter, moreover, offered an explanation which harmonized perfectly with the amboceptor theory. Bordet and Gay themselves failed when they attempted this crucial experiment. From the fact that guinea-pig blood-cells which have been treated with horse serum are strongly agglutinated by inactive ox serum, they conclude, however, that a binding of the "colloid" has occurred. We should like to point out that hæmolysis and agglutination cannot be regarded as due to one and the same substance, and that consequently there is no justification for the conclusion drawn by these authors concerning the hæmolytic constituent of ox serum. Bordet and Gay, to be sure, seek to explain the failure attending this important (for their conception) experiment by regarding the absence of hæmolysis as due to a marked antagonistic effect exerted by the strong agglutination. They found that such agglutinated blood-cells would not dissolve even when they were resuspended in a fresh mixture of inactive ox serum and horse serum. We look in vain, however, for an experiment which would have decided the question absolutely. Thus, if the guinea-pig blood-cells digested with horse serum really do absorb the hæmolytic component of ox serum, it should be possible to show that the ox serum has lost the power to dissolve guinea-pig blood in conjunction with horse serum. Bordet and Gay do mention that inactive ox serum which

had been treated with sensitized ox blood previously digested with horse serum does dissolve guinea-pig blood in conjunction with horse serum less rapidly and less actively than does native (i.e., untreated) ox serum. We should imagine that, according to the views of Bordet and Gay, the ox serum in this case would have been completely exhausted. Be this as it may, the fact remains that the one decisive experiment has not been made.

## II.

In our experiments, therefore, we first sought to fill this gap. We made use of 5% suspensions of guinea-pig blood-cells, which, of course, were washed free of serum. The ox serum was inactivated by half an hour's heating to 53-54°. In all the tests the mixtures were brought up to the same volume with physiological salt solution, and this volume was never less than 2 cc. nor more than 2.3 to 2.5 cc. The titration of the horse serum is shown in the following table.

TABLE I.

Amount of Active Horse Serum. cc.	Hæmolysis of 1 cc. 5% Guinea-pig Blood by Means of Horse Serum.	
	A On the Addition of 0.1 cc. Inactive Ox Serum.	B Without any Further Addition.
0.5	complete	}
0.35	complete	
0.25	almost complete	
0.15	moderate	
0.1	little	
0.05	trace	
0	0	0

After this to each 1 cc. 5% suspension guinea-pig blood was added 0.35 cc. horse serum, i.e., sufficient to surely activate, and the mixtures digested at 37° for one hour. A test of the decanted fluids showed that the active principle had been bound by the blood-cells. (See Table II.)

The blood sediments which had thus been treated with horse serum were next digested for one hour at 37° with decreasing amounts of inactive ox serum. No hæmolysis occurred. The tubes were then centrifuged and the decanted fluids digested with 0.35 cc. horse serum plus the sediment from 1 cc. 5% guinea-pig blood. (Series A.)

TABLE II.

Amount of Inactivated Ox Serum. cc.	Hæmolysis of 1 cc. 5% Guinea-pig Blood by Inactivated Ox Serum Plus 0.35 cc. Horse Serum.	
	A Native Horse Serum.	B Horse Serum Digested with Guinea-pig Blood.
0.25	complete	trace
0.15	"	faint trace
0.1	"	0
0.05	moderate	0
0.025	trace	0
0	0	0

In a control series (Table II, B), the sediment from 1 cc. 5% guinea-pig blood was mixed with 0.35 cc. horse serum plus the decanted fluids from 1 cc. 5% guinea-pig blood. The latter had also been digested with decreasing amounts of inactive ox serum, without, however, having previously been treated with horse serum. The result is shown in Table III.

TABLE III.

Amount of Inactive Ox Serum. cc.	Hæmolysis of 1 cc. 5% Guinea-pig Blood by 0.35 cc. Horse Serum and Inactive Ox Serum.	
	A Ox Serum Treated with Blood Pre- Digested with Horse Serum.	B Ox Serum Treated with Native Blood.
0.25	complete	complete
0.15	"	"
0.1	almost complete	almost complete
0.05	moderate	moderate
0.025	trace	trace
0	0	0

The table clearly shows that the guinea-pig blood does not absorb the active principle of the ox serum even when the blood is first digested with horse serum. We were able to confirm the result by repeating the experiment several times. The assumption of Bordet and Gay, according to which the hypothetical colloid of ox serum (the carrier of the hæmolytic action) is bound by blood-cells which have been digested with horse serum, is thus shown



to be incorrect. We may add that we too observed marked agglutination on adding ox serum to the guinea-pig blood previously treated with horse serum. Nevertheless, on testing the ox serum separated by centrifuge, we found that this still possessed all its power to effect hæmolysis of guinea-pig blood in conjunction with horse serum.

By this we do not intend to combat the statements of Bordet and Gay, that guinea-pig blood treated successively with horse serum and inactive ox serum is resistant to the hæmolytic action of the active mixture. We too have made similar observations, though we noted that hæmolysis was absent only when the guinea-pig blood-cells had been treated with an excess of horse serum. Under these circumstances it was immaterial whether the previous treatment was only with horse serum or whether treatment with horse serum was followed by digestion with inactive ox serum. It is to be noted, however, that even when the guinea-pig blood-cells were found resistant, there was no absorption of the hæmolytic component of the ox serum.

In the following experiment, which illustrates the conditions just described, we first determined the minimum amounts of active horse serum and inactive ox serum which, combined, just sufficed to produce complete hæmolysis. This dose was found to be 0.25 cc. for each.

Two parallel series were prepared. To 1 cc. 5% guinea-pig blood were added decreasing amounts of active horse serum. The mixtures were kept at 37° for one hour, and then centrifuged.

Series A. The sediments of series A were digested with 0.25 cc. active horse serum and 0.25 cc. inactive ox serum, the whole being made up to about 2.25 cc. with physiological salt solution.

Series B. The sediments of series B were once more digested for one hour at 37° with 0.25 cc. inactive ox serum (and salt solution). The mixtures were then centrifuged and the sediments thus obtained mixed with 0.25 cc. horse serum plus 0.25 cc. inactive ox serum.

Series C. The supernatant fluids separated by centrifuge in series B were mixed with guinea-pig blood and with 0.25 cc. horse serum. (Total volume about 2.25 cc.)

The result is shown in Table IV.

The table shows that the guinea-pig blood-cells do not lose their normal susceptibility when they are treated with a dose of horse serum sufficient to produce complete hæmolysis (0.25 cc.).

TABLE IV.

Amount of Horse Serum Used for Treating the Blood-cells. cc.	Hæmolysis of 1 cc. 5% Guinea-pig Blood in		
	Series A.	Series B.	Series C.
1.0	trace	trace	complete
0.5	moderate	moderate	"
0.25	complete	complete	"
0.15	"	"	"
0.1	"	"	"
0	"	"	"

It also shows that an excess of horse serum gives rise to an increased resistance of the blood-cells toward what is otherwise a hæmolytic mixture, and this effect is produced whether or not ox serum is subsequently allowed to act on the cells. Moreover, from Column C, we see that in this case also the ox serum has not lost its ability to produce hæmolysis in conjunction with horse serum. Concerning the cause of the resistance produced by treating guinea-pig blood-cells with horse serum alone or with horse serum and ox serum, we can only conjecture. It is quite possible that the effect is due to an antagonism between agglutination and hæmolysis, as suggested by Bordet and Gay. It is also conceivable that horse amboceptor and ox amboceptor attack the same receptors of the blood-cells, and that preliminary treatment with an excess of horse serum blocks the way for the ox amboceptor. Be this as it may, our experiments show that the ox serum component concerned in this hæmolysis is not bound when the guinea-pig blood-cells are first digested with active horse serum. Bordet and Gay's assumption, that ox serum produces this hæmolysis through a "colloid" constituent which acts only after amboceptor and complement have combined with the guinea-pig blood-cell, must therefore be abandoned. On the other hand, Klein's observation, that guinea-pig blood previously treated with horse serum is no longer dissolved by inactive ox serum, is readily explained in accordance with the ideas expressed by Browning. The horse serum complement, though not dominant for the horse amboceptor, is anchored to the cell by means of this amboceptor, and thus is no longer available for the ox amboceptor. We have tried to illustrate the conditions in Figs. 1 and 2 of the accompanying plate. It may be added that in this case it is impossible to produce hæmolysis either

by employing an excess of ox serum, or by employing the minimum complete solvent dose of horse serum for the preliminary treatment of the guinea-pig blood-cells. In contrast to this, the blood-cells which have been previously treated with horse serum only then fail to hæmolyze on the addition of ox serum plus horse serum when the amount of horse serum used for the preliminary treatment is excessive. As we have seen, Bordet and Gay regarded the resistance of the cells against ox serum alone and against the combined action of ox serum and horse serum as having a common origin. From what has been said it is apparent, however, that these phenomena will have to be considered separately. In the former case complement is absent, and the inhibition is therefore absolute. In the latter case complement is present, the absence of hæmolysis being a secondary effect dependent on quantitative relations. In the case described by Ehrlich and Sachs, in which guinea-pig blood is hæmolyzed by inactivated ox serum and horse serum, we do not see the least reason for abandoning the explanation offered by the authors. According to this, it will be remembered, the amboceptor is contained in the ox serum. In this combination it is absolutely unnecessary to assume the existence of a third component which takes part in the hæmolytic action.

Nevertheless we sought to find additional evidence to show that the two components of horse serum and inactive ox serum were directly related to one another, or that the amboceptor contained in horse serum played no part in the hæmolysis. In this we were successful in more ways than one. If it is necessary for ox amboceptor and horse complement to first unite and form an active hæmolysis before combining with the cell receptors, we should expect that hæmolysis would result more quickly if horse serum and ox serums were digested for a time before adding the blood, than if all three components were mixed at once. We therefore proceeded as follows:

Two series of tubes were prepared:

*Series A.* Decreasing amounts of horse serum (total volume 0.75 cc.) were kept for one hour at 37°. Then 1 cc. 5% guinea-pig blood plus 0.5 cc. inactive ox serum are added to each tube.

*Series B.* Decreasing amounts of horse serum are digested for one hour at 37° with 0.5 cc. inactive ox serum, after which 1 cc. 5% guinea-pig blood is added to each tube.

The degree of hæmolysis was noted at the end of 5, 15, and 30 minutes and after two hours. The result is shown in Table V.

TABLE V.

Amount of Horse Serum. cc.	Degree of Hæmolysis.							
	Series A.				Series B.			
	5 Min.	15 Min.	30 Min.	2 Hrs.	5 Min.	15 Min.	30 Min.	2 Hrs.
0.75	0	0	strong	complete	strong	complete	complete	complete
0.5	0	0	moderate	"	"	{ almost complete	"	"
0.35	0	0	slight	strong	0	moderate	{ almost complete	"
0.25	0	0	0	slight	0	0	strong	strong
0.15	0	0	0	trace	0	0	slight	slight
0.1	0	0	0	faint trace	0	0	0	trace
0.05	0	0	0	0	0	0	0	faint trace
0	0	0	0	0	0	0	0	0

The table shows that hæmolysis is actually more rapid when horse serum and inactive ox serum are first allowed to remain in contact for a time. During this time the ox amboceptor and horse complement have entered into combination, and the period of incubation preceding hæmolysis is thus shortened. Moreover, as can be seen from the table, the final hæmolytic effect may also be somewhat greater when amboceptor and complement are first digested together. The reason for this evidently lies in a slight impairment of the horse complement as a result of the one hour's heating to 37°, the combination of ox amboceptor and horse complement evidently being more resistant. It need hardly be mentioned that these results are incompatible with the colloid theory.

If we could remove the amboceptors of horse serum it would be possible to demonstrate directly the amboceptor rôle played by the ox serum. It is well known that a method devised by Ehrlich and Morgenroth<sup>1</sup> enables us to separate the amboceptor and complement of an active serum. Thus, by digesting red blood-cells at 0° with an active serum, it will be found that only amboceptor has been bound; the complement remains in the fluid. In the case of the normal hæmolysins, to be sure, a difficulty arises from the fact that the binding of amboceptor at 0° is usually incomplete, some of the amboceptor remaining unbound. So in the case of the amboceptors of horse serum, we know from the work of Browning that at 0° guinea-pig blood-cells bind them only up to

<sup>1</sup> Ehrlich and Morgenroth, Berliner klin. Wochenschr., 1899. See also this volume, page 1.

a certain point. The portion bound, to be sure, is not inconsiderable. It is to be noted, however, that horse serum treated with guinea-pig blood at 0° loses practically none of its power to effect hæmolysis in conjunction with inactive ox serum. According to Bordet and Gay's conception, provided that any considerable quantity of amboceptor had been bound, this should not be the case, for in the opinion of these authors the horse serum plays the rôle of amboceptor in the hæmolysis. A decrease in the quantity of amboceptor should, of course, manifest itself by a reduction in the hæmolytic power. It might be objected that the amboceptor in horse serum exists in excess, and that therefore it was entirely irrelevant whether a portion was present or absent. This objection, however, can be tested experimentally. Suppose, for example, that the horse serum digested at 0° with guinea-pig blood, still contained enough amboceptor to produce, in conjunction with inactive ox serum, the full hæmolytic effect as conceived by Bordet and Gay. It is obvious that when such a serum is subsequently treated with guinea-pig blood at 37° the impairment in the ability to bring about hæmolytic effects should be as great or even greater than that produced in native horse serum. The experiment, however, shows that just the contrary is the case. The conditions are really reflected in Table 2 of Browning's paper. We shall, however, reproduce the result of an analogous experiment.

Three series of tubes are prepared, each containing 1 cc. 5% guinea-pig blood and decreasing amounts of horse serum diluted with the same amount of physiological salt solution. The total volume in each tube is 2 cc.

The tubes of series A are kept at 37° for 1½ hours and then centrifuged. The supernatant fluids thus obtained are then digested with the sediments from 1 cc. 5% guinea-pig blood plus 0.3 cc. inactive ox serum.

The tubes of series B are centrifuged after having been kept at 0° for two hours. The supernatant fluids are treated as is series A.

The tubes of series C are centrifuged after having been kept at 0° for two hours. The supernatant fluids are digested for two hours at 37° with the sediments from 1 cc. 5% guinea-pig blood. After again centrifuging, the supernatant fluids are treated with the sediments from 1 cc. 5% guinea-pig blood plus 0.3 cc. inactive ox serum.

The result is shown in Table VI.

The horse serum which underwent a preliminary treatment at 0° is thus seen to have lost but little of its power to bring about hæmolysis, by the subsequent digestion at 37°. Certainly the reduction is considerably less than that produced by the direct

TABLE VI.

Amounts of the Half-diluted Horse Serum. cc.	Hæmolysis of 1 cc. 5% Guinea-pig Blood by 0.3 cc. Inactive Ox Serum and Horse Serum.			
	Native.	Previously Treated with Guinea-pig Blood		
		A. at 37° C.	B. at 0° C.	C. at 0° + 37°
0.5	complete	almost complete	complete	complete
0.25	"	strong	"	"
0.15	"	moderate	"	almost complete
0.1	"	trace	strong	moderate
0.05	strong	faint trace	moderate	slight
0.025	moderate	0	slight	trace
0.0	0	0	0	0

treatment of the native serum at 37°. This is all the more noticeable because in the above table a slight reduction of hæmolytic power is shown as a result of digestion at 0°. This reduction is probably due to a slight loss of supernatant fluid in decanting the centrifugates. The result of the experiment is absolutely at variance with the colloid theory. Assuming that the horse serum acts both as amboceptor and complement, while the ox serum, in accordance with the view of Bordet and Gay, furnishes a "colloid" which takes part in the hæmolysis, it follows that successive treatment at 0° and 37° would effect a greater reduction of the active principle than a single treatment at 37°. The result, on the other hand, harmonizes perfectly with the view expressed by Ehrlich and Sachs, and could, in fact, have been foretold on the basis of that conception. The horse serum furnishes only the complement. By treatment at 0° a portion of the amboceptor is removed, so that the serum thus becomes rich in complement but poor in amboceptor. On digesting such a serum once more with guinea-pig blood, at 37°, a small amount of complement is removed through the intervention of what amboceptor still remains. The loss of complement thus sustained is bound to be less than that observed when native serum (which is rich in amboceptor) is digested with guinea-pig blood. Our experimental analysis therefore shows that the interpretation offered by Bordet and Gay cannot be harmonized with the facts. In fact our study furnishes additional confirmation for the view that in the case under discussion the ox serum acts as an amboceptor with the horse serum as complement.

## III.

Our further efforts had, naturally, to be directed to a study of the experiment reported by Bordet and Gay which forms so important a link in their demonstration. It is based on the unique observation that ox blood laden with specific amboceptor does not dissolve in horse serum, but does so in a mixture of active horse serum and inactive ox serum. It is true that there is a certain external analogy between this phenomenon and the hæmolysis of guinea-pig blood by the same mixture. In the hæmolysis of the sensitized ox blood it is impossible that the ox serum acts as amboceptor, and this leads Bordet and Gay to conclude that in the hæmolysis of the guinea-pig blood the ox serum does not act as an amboceptor. We have already seen that this conclusion is not warranted. It was felt, however, that it would be interesting to inquire more closely into the peculiar mechanism of the hæmolytic action in the ox-blood combination, the more so since the view of Bordet and Gay, that the ox serum represents a "colloid" which dissolves the blood-cells previously prepared by amboceptor and complement, is an assumption devised for this particular case, and one which would constitute an entirely new kind of serum hæmolysis. We therefore sought to find an explanation for the hæmolysis in question on the basis of phenomena previously observed.

In our experiments we used an inactivated immune serum derived from a rabbit which had been immunized with ox blood. One cubic centimeter 5% ox blood was just completely dissolved (in the presence of 0.1 cc. guinea-pig complement) by 0.0005 cc. of this specific immune serum. In order to effect hæmolysis of ox blood by the mixture "horse serum plus inactive ox serum" it was necessary to use 0.05 cc. amboceptor. In the following experiment, when speaking merely of prepared ox blood, it is understood that 1 cc. 5% ox blood was treated with 0.05 cc. amboceptor. Amounts smaller than this did not suffice for complete hæmolysis, and larger amounts had to be avoided because then even small amounts of horse serum alone would produce hæmolysis. In fact according to our experience the prepared blood-cells are often hæmolyzed to a greater or less extent by the horse serum alone when this is used in rather large doses. This frequently makes it impossible to determine the dose of horse serum, which by itself is inert but which in

conjunction with inactive ox serum still produces complete hæmolysis. Herein we see the first difference between this hæmolysis and the hæmolysis of guinea-pig blood, for in the latter the horse serum was always inert or only feebly hæmolytic. Moreover, he have encountered further marked differences which speak strongly against the identity of the mechanism in the two cases which Bordet and Gay cite as analogous. Thus it was found that an excess of ox serum inhibits the hæmolysis of the prepared *ox-blood* cells by horse serum plus ox serum, whereas the degree of hæmolysis of the *guinea-pig blood* cells is proportionate to the amount of ox serum. This is shown in the following experiment:

Two series of tubes were prepared:

*Series A.* One cc. prepared 5% ox blood plus decreasing amounts of inactive ox serum plus 0.15 cc. horse serum (minimum amount).

*Series B.* One cc. 5% guinea-pig blood plus decreasing amounts of inactive ox serum plus 0.25 cc. horse serum (minimum amount).

The degree of hæmolysis is shown in Table VII.

TABLE VII.

Amount of Inactive Ox Serum. cc.	Series A.	Series B.
1.0	slight	complete
0.5	moderate	"
0.25	almost complete	"
0.1	complete	"
0.05	moderate	moderate
0.025	slight	slight
0.01	trace	trace
0	0	0

The behavior of the ox serum in the two series is totally different, so that it is impossible to ascribe the action of the serum to one and the same cause. According to Bordet and Gay, however, the ox serum in both cases acts neither as amboceptor nor as complement, but participates in the reaction as a colloid as already discussed. From this standpoint it is impossible to understand the difference in the behavior of the ox serum in the two series. Looked at from our point of view, however, the difference is readily explained, for then we regard the ox serum as acting as an amboceptor in the



hæmolysis of guinea-pig blood, but acting in quite another manner in the hæmolysis of the prepared ox blood.

Another difference between the two phenomena is presented by the following: If prepared ox blood-cells are successively digested with horse serum and inactive ox serum, no hæmolysis occurs. This is entirely analogous to what is observed with guinea-pig blood-cells. While, however, when a large amount of horse serum has been used, the guinea-pig blood-cells are resistant to the combined action of horse serum and inactive ox serum, this is not the case with the prepared ox blood. Before going into details, however, it may be well to make certain general observations concerning the behavior of the components in the hæmolysis of prepared ox blood. Thus it was found that to be impossible to replace the inactive ox serum by hog or rabbit serum. The same was true for inactive sheep serum,<sup>1</sup> whereas inactive goat serum in conjunction with horse serum acted like ox serum though weaker.<sup>2</sup> We also noted the effect of thermic influence on the components of horse serum<sup>3</sup> and found that the ox serum could be heated for half an hour to 55° without affecting its action, while on heating for half an hour to 65° it lost its power to dissolve prepared ox blood in conjunction with horse serum. So far as the relation of the individual components to the prepared blood-cells is concerned, it was found that active horse serum is robbed of its active constituent by treatment with prepared blood. In fact, not only does it thereby lose its property to dissolve prepared ox blood (confirming Bordet and Gay), but it also ceases to dissolve guinea-pig blood in conjunction with inactive ox serum (confirming the statements of Browning). This was to be expected, because in both combinations the horse serum acts as complement, and a suitable amboceptor is present. In both cases, therefore, the amboceptor can effect absorption of complement without giving rise to hæmolysis. There is another point of agreement between the two combinations. Thus, despite the anchoring of horse complement brought about by treatment with horse serum, the prepared ox blood-cells do not dissolve on the addition of inactive ox serum.

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<sup>1</sup> Active sheep serum by itself is slightly hæmolytic for prepared ox blood. The action is intensified, however, by the addition of horse serum.

<sup>2</sup> It should be remarked that in the hæmolysis of guinea-pig blood the ox serum can be replaced by goat serum. The mode of action is the same in both cases.

<sup>3</sup> Ox serum?—[Editor.]

Prepared blood so treated, however, at once dissolves in a mixture containing minimum quantities of horse serum and inactive ox serum. This is illustrated in the following experiment:

Two similar series of tubes are prepared. The tubes in each series contain 1 cc. 5% prepared ox blood and decreasing amounts of active horse serum (total volume 2 cc.). After remaining for two hours at 37° the tubes are centrifuged. In the first two, containing the largest amounts of horse serum, a trace of hæmolytic was noticed.

A. The supernatant fluids were mixed each with the sediments of 1 cc. 5% prepared ox blood, plus 0.1 cc. inactive ox serum. (0.1 cc. is the smallest dose necessary to produce complete hæmolytic.)

B. The sediments are suspended in salt solution plus 0.1 cc. inactive ox serum.

C. The sediments are suspended in salt solution plus 0.1 cc. inactive ox serum plus 0.35 cc. horse serum.

The result is shown in the following table:

TABLE VIII.

Amount of Horse Serum. cc.	Degree of Hæmolytic of 1 cc. 5% Prepared Ox Blood plus 0.1 cc. Inactive Ox Serum plus Horse Serum.			
	Control.	A.	B.	C.
1.0	complete	slight	0	complete
0.5	"	trace	0	"
0.35	"	faint trace	0	"
0.25	strong	0	0	"
0.15	moderate	0	0	"
0.1	trace	0	0	"
0	0	0	0	"

Column B of the table shows exactly the same behavior as in a corresponding experiment with guinea-pig blood. Despite the fact that horse serum has bound the amboceptor and complement, there is no hæmolytic on the addition of inactive ox serum. One of the main arguments which could have been advanced in support of Bordet-Gay's colloid theory thus fails. It is also apparent that no special resistance of the prepared blood-cells comes into question, for in column C we find that these cells are completely dissolved in a suitable mixture.

Bordet and Gay, to be sure, do say that prepared ox blood-cells treated with horse serum absorb the effective principles of inactive horse serum. However, all that they describe as a result of this is a

marked agglutination; they say nothing about the occurrence of hæmolysis, though hæmolysis is what one should have expected according to their theory. On the other hand the authors tell us that ox serum, by acting on ox blood which has been prepared and loaded with horse complement, loses its power to *agglutinate*, in conjunction with horse serum, prepared ox blood. Nothing is said about hæmolytic action. According to the authors ox serum so treated when tested in conjunction with horse serum on guinea-pig blood, does agglutinate and dissolve the blood more slowly and more feebly. We felt it advisable to study the conditions more closely, and proceeded along the lines already described in our analysis of guinea-pig blood hæmolysis.

Two series of tubes are prepared. Each tube contains 1 cc. prepared 5% ox blood which has previously been treated for one hour with 0.5 cc. horse serum (=2 complete hæmolytic doses) at 37° and then freed from fluid by centrifuge. Decreasing amounts of inactive ox serum are added to each tube, the mixtures kept at 37° for one hour and centrifuged. The decanted fluids in the one series are digested each with the sediments from 1 cc. 5% prepared ox blood plus 0.25 cc. horse serum, and in the other series with the sediments from 1 cc. 5% guinea-pig blood plus 0.25 cc. horse serum. The result is shown in the following table:

TABLE IX.

Amount of Inactive Horse Serum.	Hæmolysis of			
	1 cc. 5% Prepared Ox Blood by 0.25 cc. Horse Serum plus		1 cc. 5% Guinea-pig Blood by 0.25 cc. Horse Serum plus	
	A. Treated Ox Serum.	B. Native Ox Serum.	A. Treated Ox Serum.	B. Native Ox Serum.
cc.				
0.35	complete	complete	complete	complete
0.25	"	"	almost complete	almost complete
0.15	"	"	strong	"
0.1	strong	strong	slight	moderate
0.05	moderate	moderate	faint trace	trace
0	0	0	0	0

From the table it can be seen that ox blood loaded with horse complement is likewise unable to deprive inactive ox serum of the constituent which brings about hæmolysis. In fact ox blood so treated is able, in conjunction with horse serum, to dissolve with full or only slightly impaired power not only prepared ox blood-cells

but also those of the guinea-pig. In this respect, therefore, our results are somewhat opposed to the statements of Bordet and Gay. For the sake of completeness it may be mentioned that ox serum digested with guinea-pig blood which has previously been treated with active horse serum loses nothing of its power to bring about hæmolysis of prepared ox blood.

To sum up: 1. Prepared ox blood treated with active horse serum does not dissolve in inactive ox serum.

2. The constituent of the ox serum which brings about hæmolysis is not absorbed by prepared ox blood previously treated with horse serum.

This shows that the hæmolysis of prepared ox blood by the combined action of inactive ox serum and active horse serum, as also the hæmolysis of guinea-pig blood under the same conditions cannot be explained on the basis of the colloid theory of Bordet and Gay. We have seen that the simplest postulates of this theory cannot be verified experimentally. In the hæmolysis of guinea-pig blood it is at once clear that it is not the horse serum, as Bordet and Gay suppose, but the ox serum which furnishes the hæmolytic amboceptor. This ox amboceptor, as Ehrlich and Sachs have shown, is peculiar in that it requires first to be united with horse complement before it can be anchored by the red blood-cells.

In explaining the hæmolysis of prepared ox blood, it is impossible to regard the ox serum as acting as an amboceptor, and Bordet and Gay have very properly called attention to this fact. One might perhaps think that the inactivated ox serum acts as a complementoid. In that case, to be sure, the function of the complementoid would be rather peculiar. It would be necessary to assume that the active horse complement was bound by the amboceptor-laden blood-cells at an unsuitable point so that the complement could not exert its action, or, in other words, so that it was "not dominant." The rôle of the ox complementoid would then consist in directing, as it were, the horse complement in the right direction. One could, for instance, imagine that the complementoid possessed a higher affinity than the horse complement, and that it would thus block the amboceptor group at which the complement is not dominant. The horse complement would thus be anchored by the complementophile amboceptor group for which it really possesses the smaller affinity but at which it is dominant. Still other interpretations are possible, but it would always be necessary to assume that the ox complementoid

is already itself bound by the prepared ox blood. It can, however, be shown that the active principle of the ox serum loses none of its power by digestion with prepared ox blood-cells. From this it follows that the view just discussed, wherein the ox serum is regarded as acting as a complementoid, is incorrect.

It was necessary to cast about for other explanations, and it was natural to think that in the hæmolysis of the prepared ox blood too, the inactive ox serum possessed direct relations to the horse serum. We had noticed that the ox serum amboceptor acting on guinea-pig blood possessed a marked affinity for horse complement. This fact suggested that the ox serum could produce anticomplementary effects, for it is readily understood that an amboceptor possessing affinity for the complement will act like an anticomplement when the suitable blood-cells are absent. As a matter of fact we have shown (see Table VII) that large amounts of inactive ox serum hinder the hæmolysis of the prepared ox blood. This inhibition can only be due to anticomplement action. These findings naturally led us to suspect that the inactive ox serum and the horse serum were in some way related to one another in the production of the hæmolytic effect. We therefore proceeded as follows:

In one series of tubes decreasing amounts of horse serum were kept for one hour at 37°, whereupon prepared ox blood plus 0.5 cc. inactive ox serum were added. In another series decreasing amounts of horse serum were digested for one hour with 0.5 cc. inactive ox serum at 37° whereupon the blood-cells were added. The degree of hæmolysis was noted from time to time, and is shown in the following table:

TABLE X.

[illegible]

The table shows the same condition which we have already noted in the hæmolysis of guinea-pig blood. The hæmolysis of the prepared ox blood too, proceeds more rapidly if the horse serum and inactive ox serum are mixed some time before the addition of the blood-cells. From this it follows that some sort of a reaction takes place between constituents of the horse serum and of the ox serum. A really active complex as in the hæmolysis of guinea-pig blood cannot thus be formed, for, as we have repeatedly pointed out, the ox serum cannot functionate as an amboceptor. We shall probably not err if we assume that the inactive ox serum participates in the hæmolysis of the prepared ox blood by anchoring a constituent of horse serum which inhibits the action of the horse complement responsible for hæmolysis. An autoanticomplement of horse serum is out of the question, if only for the reason that the horse complement is bound by the prepared ox blood-cells. On the other hand it seemed very possible that the horse serum constituent in question which inhibits hæmolysis and which is bound by ox serum, possessed the character of a complement or a complementoid. The action of this second complement of horse serum would be this, that it does not dissolve prepared ox blood, but possesses a higher affinity than the effective complement. The anchoring of this constituent would cause the effective complement to be bound at an unsuitable situation where it is not dominant. In order to prove the correctness of this view it is necessary to show that the binding of the effective horse complement to the prepared ox blood, and the hæmolysis of prepared ox blood by the joint action of active horse serum and inactive ox serum, are two independent reactions. In other words we must effect a binding of the active principle of horse serum and yet have no hæmolysis when under exactly the same conditions inactive ox serum is also present. This we have succeeded in doing. It is very easy to fulfil the conditions just mentioned, by digesting the ox blood with a smaller quantity of amboceptor. We proceeded as follows:

Two series of tubes are prepared, each tube containing 1 cc. 5% ox blood and decreasing amounts of amboceptor (inactivated serum of a rabbit immunized against ox blood). After remaining at 37° for one hour, the mixtures were centrifuged. The sediments were then treated as follows:

*Series A.* Digested with a mixture of 0.2 cc. horse serum plus 0.1 cc. inactive ox serum.

*Series B.* Digested with 0.2 cc. horse serum<sup>1</sup> for one hour at 37°, centrifuged, and the sediments thus obtained mixed each with 0.1 cc. inactive ox serum.

*Series C.* The supernatant fluids separated in B are digested with the sediments each of 1 cc. 5% prepared ox blood (prepared in the usual way with 0.05 cc. amboceptor) plus 0.1 cc. inactive ox serum.

The result is shown in the following table:

TABLE XI.

Amount of Amboceptor Used for the Preliminary Treatment.  cc.	Degree of Haemolysis.		
	Series A. More or Less Highly Prepared Ox Blood + 0.2 cc. Horse Serum + 0.1 cc. Inactive Ox Serum.	Series B. More or Less Highly Prepared Ox Blood + 0.2 cc. Horse Serum Centrifuged, + 0.1 cc. Inactive Ox Serum.	Series C. Highly Prepared Ox Blood (0.05) + 0.1 cc. Inactive Ox Serum + 0.2 cc. Previously Digested Horse Serum
0.1	complete	0	faint trace
0.05	"	0	"
0.025	strong	0	"
0.015	slight	0	"
0.01	trace	0	"
0.005	0	0	"
0.0025	0	0	slight
0	0	0	complete

Total volume always 2 cc.

The table shows that so far as the binding of horse complement is concerned, ox serum which has been prepared with one-tenth the amount of amboceptor (0.005 cc.) behaves exactly the same as that which has been highly prepared (0.05 cc. amboceptor). In spite of this, we see that such feebly prepared ox blood is resistant to the combined action of horse serum and inactive ox serum (Series A). Furthermore, from Series B it is apparent that the successive addition of horse serum and inactive ox serum does not lead to haemolysis. The conditions discussed above have thus been fulfilled, and the result shows that the phenomenon of the binding of horse complement must be considered apart from that of its haemolytic action.

The following is probably the simplest conception we can make of the mechanism of the entire phenomenon. In view of the multiplicity of amboceptors in a given immune serum (see especially the studies of Ehrlich and Morgenroth) there is no reason why we should not be dealing with two different fractions of amboceptor in the immune serum used to prepare the ox blood. One amboceptor is present in high concentration and binds the horse complement, although the complement is not dominant for this amboceptor.

The other amboceptor is present in much smaller amount, and is the amboceptor for which the horse complement is dominant. This explains how a small amount of amboceptor binds complement, and how hæmolysis is effected only with a considerable excess of immune serum. The relations existing between weak and strong concentration of amboceptor in the immune serum are to a certain extent analogous to those existing between horse and ox amboceptor in the hæmolysis of guinea-pig blood. There is, however, an important difference. In the hæmolysis of guinea-pig blood the affinity of the ox amboceptor to the horse complement exceeds that of the horse amboceptor. When both amboceptors are present, therefore, hæmolysis occurs. In the hæmolysis of the prepared ox blood, however, it is not sufficient that both amboceptors are present, for under these circumstances, apparently, the complement is still anchored by the amboceptor for which it is not dominant. In order that the complement may lay hold of the other amboceptor, the coöperation of the inactive ox serum is necessary. This serum, as we have seen, must have direct relations with the horse serum. The only way in which we can conceive of this relation is to assume that the ox serum binds a horse serum constituent of complement character which directs the effective horse complement toward the amboceptor unsuited for producing hæmolysis. The principle underlying this explanation is not new, similar relations having been studied by Ehrlich and Marshall.<sup>1</sup> In a combination described by these authors, it was shown that the union of a certain non-dominant complement did not occur until after another complementophile group of the amboceptor had bound the particular complement which was dominant in this case. It is possible that we are here dealing with an analogous phenomenon.

If we succeed, therefore, in removing the constituent of horse serum which causes the effective horse complement to combine with the unsuited amboceptor (and this, as we have seen, is accomplished by the action of ox serum), we permit the horse complement to unite with the other, effective, amboceptor and hæmolysis can occur. In this case, however, it follows that the binding of the horse complement to the weakly prepared ox blood will not occur if the horse serum constituent which brings about this binding is rendered

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<sup>1</sup> Ehrlich and Marshall, *Berliner klin. Wochenschrift*, No. 25, 1902. See also this volume, page 226.



inert by the ox serum. This we were actually able to prove experimentally. Constituting as it does the crucial experiment for testing the correctness of the views here developed, the following experiment deserves the closest attention.

Two series of tubes are prepared:

*Series A.* Each tube contains 0.35 cc. horse serum made up to 1.1 cc. with salt solution. The mixtures are kept at 37° for half an hour, and then digested for 1½ hours at 37°, each with the sediments from 1 cc. 5% weakly prepared (0.005 cc. amboceptor) ox blood. Then centrifuge. The decanted fluids are mixed with decreasing amounts of inactive ox serum (1 cc. volume) and these mixtures are poured each over the sediments from 1 cc. 5% strongly prepared (0.05 cc. amboceptor) ox blood.

*Series B.* Each tube contains 0.35 cc. horse serum plus decreasing amounts of inactive ox serum (total volume 1.1 cc.). After remaining at 37° for half an hour the mixtures are digested for 1½ hours at 37°, each with the sediments from 1 cc. 5% weakly prepared (0.005 cc. amboceptor) ox blood. After centrifuging, the decanted fluids are poured each over the sediments from 1 cc. strongly prepared (0.05 cc. amboceptor) 5% ox blood and 1 cc. salt solution is added.

The result is shown in the following table:

TABLE XII.

Amount of Inactive Ox Serum. cc.	Hemolysis of 1 cc. 5% Strongly Prepared Ox Blood.	
	Series A. By Ox Serum, and Horse Serum which has been Treated with Weakly Prepared Blood.	Series B. By Mixtures of Ox Serum and Horse Serum after the Mixtures had been Treated with Weakly Prepared Blood.
0.75	trace	complete
0.5	"	"
0.35	"	"
0.25	"	strong
0.15	"	moderate
0.1	"	trace
0	0	0

An examination of the table makes it clear that the horse complement is not bound to the weakly prepared ox blood when sufficient quantities of the inactive ox serum are added to the horse serum. This result shows at once how entirely untenable is the theory of Bordet and Gay. According to their view we would have every

reason to expect hæmolysis in Series B to be weaker than in Series A. Under no circumstances could it be stronger. In Series B conditions are such that the "colloid" of these authors would have every opportunity to be absorbed by the weakly prepared blood laden with complement. The result, however, is exactly the reverse, and absolutely contradicts the colloid theory. On the other hand the result is what was to be expected in accordance with our view. The table clearly shows that the ox serum hinders the binding of the horse complement by the weakly prepared ox blood. Proceeding from this fact we arrive at an understanding of the part played by the ox serum in the hæmolysis of strongly prepared ox blood by horse serum. We are dealing with rather complicated relations and we have therefore thought it wise to represent these in the attached diagram, figures 3-7.

Fig. 3 represents the constitution of the immune serum. Amboceptor *a* is present in weak concentration, while the other, amboceptor *b*, is present in strong concentration.

Fig. 4. pictures our conception of the relations existing when strongly prepared ox blood-cells are digested with horse serum. The immune serum used for preparing the blood contains two types of amboceptor, namely the strongly concentrated amboceptor *b*, and the weakly concentrated amboceptor *a*. (See Fig. 3.) The latter is the amboceptor for which the horse complement  $\alpha$ , is dominant. The horse serum, however, contains another substance having complementary properties,  $c\beta$  and this possesses marked affinity for the complementophile group  $\beta$  of amboceptor *b*. Amboceptor *b* also possesses a group  $\alpha$  which ordinarily does not react with  $\alpha$ . Through the anchoring of component  $c\beta$  to  $\beta$  the affinity of this group  $\alpha$  of amboceptor *b* is increased so that now amboceptor *b* lays hold on the effective complement  $\alpha$  with great avidity. Since, however, complement  $\alpha$  is not dominant for amboceptor *b*, no hæmolysis ensues.

Fig. 5 illustrates the action of the ox serum constituent *r*. This binds  $c\beta$ , whereby the increased affinity of group  $\alpha$  of amboceptor *b* fails to occur. This in turn causes  $\alpha$  to unite with  $\alpha$  thus giving rise to hæmolysis.

If amboceptor *a* is absent, i.e., if the ox blood has been weakly prepared, it will be understood that in the digestion with horse serum, amboceptor *b* binds  $c\beta$  and through this also  $\alpha$ . The decanted fluid is therefore unable to dissolve strongly prepared blood even when

inactive ox serum is present. (See Fig. 6 and also Table 12, A, of the text.)

Furthermore, if the weakly prepared blood, which then has only bound amboceptor *b*, is digested with the mixture of horse serum and inactive ox serum, no hæmolysis occurs because the effective amboceptor *a* is absent. Since, however, the ox component *r* binds  $c\beta$ ,  $c\alpha$  is left free. In this case if the decanted fluid is poured over strongly prepared ox blood, it will be found that hæmolysis occurs without any further addition. (See Fig. 7, and experiment Table 12, B.)

Naturally, in addition to the factors described above, the effects of mass action must be considered. Thus if a small quantity of ox serum is made to react with a great excess of amboceptor *b*, it is evident that the reaction between *b* and *c* can still take place. It will, however, be slower and less complete than when the ox serum is entirely absent. If then amboceptor *a* is present at the same time it will be understood that a portion of *c* will still find opportunity to combine with it so that hæmolysis occurs. But when amboceptor *a* is absent, that is when the ox blood is weakly prepared, *c* will still be able to combine with amboceptor *b* and the decanted fluid will have lost its hæmolytic power. This explains a point in Table 12. In the control which consisted of simple mixtures of strongly prepared ox blood, 0.35 cc. horse serum, and decreasing amounts of inactive ox serum, it was found that 0.1 cc. of the inactive ox serum still produced complete hæmolysis. In Table 12, B, on the other hand, weakly prepared ox blood deprived a mixture of 0.1 cc. inactive ox serum plus 0.35 cc. horse serum of its hæmolytic power.

Contrariwise we should expect to find the effective horse complement kept intact after digestion with weakly prepared ox blood provided the excess of inactive ox serum is allowed to act at the same time. This is well shown in the following experiment:

Two series of tubes are prepared:

*Series A.* Each tube contains 0.5 cc. weakly prepared 10% ox blood plus 0.5 cc. salt solution plus decreasing amounts of active horse serum.<sup>1</sup>

*Series B.* Each tube contains 0.5 cc. weakly prepared 10% ox blood plus 0.5 cc. inactive ox serum plus decreasing amounts active horse serum.<sup>2</sup>

The mixtures are kept for 1½ hours at 37° and then centrifuged. The slight amount of hæmolysis observable in series B is shown in Table XIII.

<sup>1</sup> Horse serum plus salt solution previously kept at 37° for one hour.

<sup>2</sup> Horse serum plus ox serum previously kept at 37° for one hour.

TABLE XIII.

Amount of Horse Serum cc.	Hæmolysis of 1 cc. 5% Weakly Prepared Ox Blood by Decreasing Amounts of Horse Serum.	
	A. By Itself.	B. Together with 0.5 cc. Inactive Ox Serum.
0.75	0	strong
0.5	0	slight
0.35	0	0
0.25	0	0
0.15	0	0
0.1	0	0
0	0	0

After this the fluid decanted from the tubes of series A are mixed each with 0.5 cc. inactive ox serum, and the fluids from series B, each with 0.5 cc. salt solution. The mixtures are then digested each with the sediments from 1 cc. 5% strongly prepared ox blood.

In control series C made at the same time, mixtures containing each 0.5 cc. inactive ox serum plus decreasing amounts of horse serum were digested at 37° for two hours, after which strongly prepared ox blood was added.

The result of the experiment is shown in the following table:

TABLE XIV.

Amount of Horse Serum. cc.	Hæmolysis of 1cc. 5% Strongly Prepared Ox Blood.		
	Series A.	Series B.	Series. C.
0.75	strong	complete	complete
0.5	slight	"	"
0.35	trace	"	"
0.25	0	almost complete	almost complete
0.15	0	strong	slight
0.1	0	moderate	trace
0	0	0	0

From the table it is clearly apparent that in the digestion with weakly prepared ox blood, the horse complement remains entirely intact provided plenty of ox serum is present, whereas by itself it is bound by the prepared blood, as can be seen from Column A. The evidence presented by this marked difference becomes still stronger through the fact that the action of mixtures of horse serum and ox serum on weakly prepared blood results in a slight degree of

hæmolysis (See Table 13, B). Despite the occurrence of this hæmolysis in which at least some material has been used up, the final result is just the opposite of what was, *a priori*, to have been expected. This furnished a weighty argument in favor of the view we have brought forward. We shall probably not err if we assume that the horse serum constituent  $c\beta$  is a complement, but that it is dominant neither for amboceptor  $a$  nor amboceptor  $b$ . The ox serum thus plays merely the part of anticomplement. The amboceptors of ox serum in general evidently possess a high affinity in their complementophile groups. It will be recalled that we have actually demonstrated this in the case of the amboceptor acting on guinea-pig blood and complemented by horse serum. A little consideration, however, will show that such amboceptors, when the cells on which they act are missing, will exert an anticomplementary action. This also explains how the inactivated ox serum when in excess, can inhibit the hæmolysis of strongly prepared ox blood by horse complement  $c\alpha$ , although this same ox serum, in smaller quantities, brings this hæmolysis about. This observation has been repeatedly made by us. It is merely necessary to assume that ox serum also contains very small quantities of complementophile groups  $\alpha$ . Large doses of the serum would then also exert a deflecting influence on complement  $c\alpha$ .

So far as the two complements of horse serum are concerned ( $c\alpha$  and  $c\beta$ ) it seems as though their quantitative relations are subject to certain fluctuations. We have already called attention to the fact that horse serum alone dissolves prepared ox blood cells to a varying degree. In the light of what has been said it is obvious that the hæmolysis produced by horse serum alone will be stronger the more the concentration of the horse complement  $c\alpha$  exceeds that of complement  $c\beta$ . If complement  $c\beta$  were entirely absent we should find that the hæmolysis produced by horse serum alone would be as strong as that produced by the combined action of horse serum and inactive ox serum. We have not met with such extreme cases. Nevertheless we have observed horse sera which by themselves produced complete hæmolysis of prepared ox blood in doses of 0.35 to 0.3 cc. while the addition of inactive ox serum reinforced complete hæmolysis only to the extent of a dose of 0.15 cc. horse serum. We see, therefore, that a critical study of the experimental findings leads to conclusions which fit perfectly into the interpretation we have elaborated.

## EXPLANATION OF THE FIGURES ON THE PLATE.

FIGS. 1 and 2 illustrate the hæmolysis of guinea-pig blood by the combined action of active horse serum and inactive ox serum.

$z$  = guinea-pig blood-cell;  $ar$  = amboceptor of ox serum;  $ap$  = amboceptor of horse serum;  $c$  = complement of horse serum.

FIG. 1 represents the conditions obtaining when blood, horse serum, and ox serum are mixed simultaneously. The ox amboceptor ( $ar$ ) combines with the horse complement ( $c$ ) and thus brings about hæmolysis.

FIG. 2.—The guinea-pig blood is first digested with horse serum ( $ap + c$ ). The blood-cell absorbs the horse amboceptor ( $ap$ ) and this in turn anchors horse complement ( $c$ ). The ox amboceptor ( $ar$ ) subsequently added does not find any horse complement ( $c$ ) at its disposal, and hæmolysis therefore does not occur.

FIGS. 3–7 illustrate the hæmolysis of ox blood laden with amboceptor, by the combined action of active horse serum and inactive ox serum.

$z$  = ox blood-cell;  $a$  and  $b$  = partial amboceptors of the immune sera ( $a$  weakly concentrated, and  $b$  strongly concentrated);  $\alpha$  and  $\beta$  = complementophile groups;  $c\alpha$  = the horse complement dominant for amboceptor  $a$ ;  $c\beta$  = the second complement-like constituent of horse serum. This is dominant neither for  $a$  nor for  $b$ ; its union, however, with amboceptor  $b$  makes the complementophile group  $\alpha$  of amboceptor  $b$  capable of reacting.  $r$  = active constituent of ox serum (anticomplement amboceptor?) which binds  $c\beta$ .

FIG. 3.—This shows the constitution of the immune serum. Amboceptor  $a$  is present in weak concentration, amboceptor  $b$  in strong concentration.

FIGS. 4 and 5 illustrate the mechanism of the hæmolysis of strongly prepared ox blood by horse serum and inactive ox serum.

FIG. 4.—Strongly prepared ox blood is digested with horse serum. Constituent  $c\beta$  of the horse serum is bound by amboceptor  $b$ , and this union causes horse complement  $c\alpha$  to combine with amboceptor  $b$ . Since  $c\alpha$ , however, is dominant only for  $a$  and not for  $b$ , no hæmolysis takes place.

FIG. 5.—Strongly prepared ox blood is digested with a mixture of active horse serum and inactive ox serum. Ox serum constituent  $r$  binds component  $c\beta$  of the horse serum, and  $c\beta$  is thus prevented from uniting with amboceptor  $b$ . Since the latter, however, does not by itself react with horse complement  $c\alpha$ ,  $c\alpha$  is bound by amboceptor  $a$  and hæmolysis is brought about.

FIGS. 6 and 7 illustrate the conditions obtaining when ox blood is prepared with a slight amount of immune serum, and when, therefore, only amboceptor  $b$  has been bound by the blood-cells.

FIG. 6.—Weakly prepared ox blood is digested with horse serum.  $c\beta$  is bound by  $b$ , and this union causes  $c\alpha$  to combine with  $b$ . No hæmolysis occurs. On centrifuging, no horse complement is found in the decanted fluids.

FIG. 7.—Weakly prepared ox blood is digested with a mixture of horse serum and inactive ox serum. Component  $r$  of the ox serum combines with  $c\beta$ . As a result of this  $c\alpha$  is not bound by  $b$ , and remains free. On centrifuging, the decanted fluid contains the horse complement.



This, we believe, disposes of the objections raised by Bordet and Gay against the view that in the hæmolysis of guinea-pig blood the ox serum constituent acts as an amboceptor. Furthermore, a thorough analysis has shown that the interpretation of Bordet and Gay is directly opposed to a number of experimental observations. In contrast to this we see that all the experimental findings fit in perfectly with the view developed on the basis of the amboceptor theory. The peculiar rôle of the ox serum is readily explained by the high affinity of the complementophile groups which the serum contains, or the high affinity of the amboceptor to the complement. This applies not only to the hæmolysis of guinea-pig blood, but also to the hæmolysis of prepared ox blood. It is unnecessary, therefore, to ascribe new and unique properties to the ox serum, as is done by Bordet and Gay. In fact the apparent exceptions to the rule are merely variations of the cytotoxic action whose occurrence can be predicated from the view developed on the basis of the amboceptor theory.

### Résumé.

1. Contrary to the view of Bordet and Gay, in the hæmolysis of guinea-pig blood by active horse serum and inactive ox serum, the amboceptor is furnished by the ox serum and not by the horse serum.

2. The guinea-pig blood absorbs the complement of horse serum through the agency of a horse amboceptor which is not dominant for the horse complement.

3. Subsequent addition of ox serum to guinea-pig blood previously treated with horse serum does not produce hæmolysis, though according to Bordet and Gay's view hæmolysis should occur. Neither is the hæmolytic component of ox serum thereby bound. This proves the incorrectness of Bordet and Gay's theory, according to which a "colloid" of ox serum constitutes a third element in the cytotoxic action, and is absorbed by the blood cells laden with amboceptor and complement, thereby effecting solution of the cells.

4. Against this a direct union of ox amboceptor and horse complement is indicated by the fact that hæmolysis is considerably more rapid when the two sera are digested before the blood-cells are added.

5. It is possible by treating the horse serum with guinea-pig blood at 0° to abstract a large part of the amboceptor without diminishing the complement content. Despite the loss of ambo-



ceptor the power of the horse serum to produce hæmolysis in conjunction with the ox serum is preserved. Moreover, when digested with blood, such a serum suffers a smaller loss of this power than does native serum. This also shows that the amboceptor bringing about hæmolysis is contained in ox serum.

6. Bordet and Gay found that ox blood loaded with amboceptor, (prepared), dissolves in a mixture of active horse serum and inactive ox serum, but not in horse serum alone. This we were able to confirm. Their interpretation, however, according to which the ox serum acts as a "colloid" in dissolving the ox blood previously prepared with horse serum, and according to which this "colloid" is bound by the prepared ox blood, this interpretation was shown to be incorrect for the following reasons:

7. Prepared ox blood absorbs the horse complement without thereby being dissolved. Blood so treated, however, does not dissolve on the addition of inactive ox serum, nor has it the power to deprive the latter of its ability to bring about hæmolysis.

8. In fact, it has been found that even in the hæmolysis of prepared ox blood inactive ox serum and horse serum stand in direct relations with each other. If both sera are digested prior to the addition of the prepared ox blood, hæmolysis will be markedly hastened.

9. Ox blood will also bind the horse complement if the blood is first treated with a small quantity of amboceptor, although hæmolysis by horse serum and inactive ox serum requires a far greater quantity of amboceptor. This shows that the immune serum contains two different amboceptors. One of these, *b*, present in high concentration, absorbs horse complement when ox serum is absent, the other, *a*, present in weak concentration, binds horse complement when ox serum is present. Only in the latter case does hæmolysis occur.

10. Ox serum prevents the binding of horse complement by weakly prepared (amboceptor *b*) ox blood, and yet does not give rise to hæmolysis in this case.

11. Since, however, the ox serum acts on the horse serum and not on the prepared blood, it follows that the ox serum binds a constituent of the horse serum, which constituent has the power to make possible and bring about the union of the horse complement and amboceptor *b*.

12. The combined action of the horse serum and inactive ox serum in the hæmolysis of prepared ox blood is thus explained by the

anticomplementary effect of the ox serum. The anticomplementary action, however, applies in the main only to a complement-like constituent of horse serum, a constituent which causes the effective horse complement to unite with an amboceptor *b*, although the complement is effective only for amboceptor *a*. The phenomenon described by Bordet and Gay, therefore, cannot be explained by their interpretation, whereas all the experimental data are easily understood on the basis of the amboceptor theory.

## XLVII. STUDIES ON ANTIAMBOCEPTORS.<sup>1</sup>

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and

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THE study of the antihæmolytic effects produced by immunization has greatly deepened in the past few years and become much more difficult. This is largely due to the recognition of the complement-binding power possessed by albuminous bodies when laden with specific antibodies. Attention was called to this phenomenon by Gengou,<sup>2</sup> who concluded that it demonstrated the existence or production of amboceptors against dissolved albuminous bodies. Moreschi,<sup>3</sup> however, deserves the credit for first directing attention to the relation of this phenomenon to the well-known anticomplementary action of the blood serum. A study of Moreschi's investigations, especially in the light of our present knowledge, makes it appear very doubtful whether the inhibiting action of immune sera formerly ascribed to the anticomplements is really due to the presence of antibodies directed against the complements, or whether it is not rather occasioned, at least in a measure, by the anticomplementary power exerted by the substance formed by the interaction of albumin and antialbumin. The problem of differentiating anticomplements *sensu strictiori* has now become more difficult than ever, because the mode of action of the anticomplements in no way differs from that of the albumin complex laden with amboceptor.

For the present, therefore, the problem of demonstrating true antihæmolytins appears to be more readily studied by directing attention first to the antiamboceptors. Our knowledge concerning

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<sup>1</sup> Reprinted from the Berlin. klin. Wochenschrift, 1906, Nos. 20 and 21.

<sup>2</sup> Gengou, Sur les sensibilatrices des sérums actifs contre les substances albuminoïdes. Annales Pasteur, 1902, T. XVI.

<sup>3</sup> Moreschi, Zur Lehre von den Anticomplementen. Berliner klin. Wochenschr., 1905, No. 37, and 1906, No. 4.

the antiamboceptors produced by immunization has undergone profound alterations within the past few years, thanks to the fundamental researches made by Bordet. These investigations were fully confirmed as to fact by Ehrlich and Sachs,<sup>1</sup> and by Muir and Browning.<sup>2</sup> We must, therefore, assume that the antiamboceptors are usually antibodies of the complementophile group, and in this respect must regard Bordet's findings as a most conclusive argument in favor of the amboceptor theory. Bordet's strongest point consists of the fact that it is possible, by immunizing with normal serum, to produce antiamboceptors which act against all the amboceptors (both normal and immune) of the species whose serum was used for immunization. But just this circumstance should arouse skepticism and make us question whether perhaps the antiamboceptor effect is not merely apparent, and counterfeited by the complement-binding power of albumin laden with antibody. The experimental analysis of this case is far more easy than the differentiation of the anti-complements. In true antiamboceptors the point of attack is a different one, being confined, as already said, to the complementophile group of the amboceptor. Nevertheless, the differentiation of the antiamboceptors is not as simple as was originally stated in Ehrlich and Morgenroth's communications. Suppose, for example, that we mix amboceptor and antiamboceptor, add blood-cells, centrifuge, wash the sediment thoroughly, and find, after the addition of complement, that hæmolysis does not take place. A little consideration will show that such a result permits of two interpretations. It may be due to an antiamboceptor; it may, however, be due to the complement-deflecting power exerted by an albuminous precipitate possibly carried down with the blood-cells laden with amboceptor. It is important to bear in mind that the serum containing the amboceptors also contains albumin antigens, and that the antiamboceptor serum contains albumin antibodies. We fully agree, therefore, with the statement made by Pfeiffer and

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<sup>1</sup> Ehrlich and Sachs, Ueber den Mechanismus der Antiamboceptorwirkung. See page 561.

<sup>2</sup> Muir and Browning, On the Properties of Anti-immune bodies and complementoids. *Journal of Hygiene*, 1906, Vol. VI, No. 1.

NOTE.—Those wishing to follow the historical development of the subject will find this discussed in the paper by Ehrlich and Sachs already alluded to. In this, too, mention will be found of the investigations of Pfeiffer and Friedberger, which may be regarded as precursors of Bordet's observations.

that "the anticomplementary action of the precipitate forfeit the existence of antiamboceptors."

An amboceptor derived from a human convalescent from Pfeiffer and Friedberger<sup>2</sup> found that bacteriolysis could be effected by a rabbit serum obtained by immunizing a rabbit with the serum. They concluded from their experiments that the possibility of this being an antiamboceptor action could be excluded, must be pointed out, however, that the results permit of no explanation. In the first place Pfeiffer and Moreschi consider it highly improbable that the antiserum obtained by immunizing a normal human serum should contain cholera antiamboceptors. This assumption is wholly unwarranted. We have already mentioned Bordet's observation that by immunizing with human serum one obtains antiamboceptors against all the amboceptors of the same species. These antiamboceptors, being directed against the complementophile group, are in their action entirely independent of the cytophilic specificity. The fact, therefore, that a normal (human) serum used for immunization contains no antiamboceptors, does not in any way argue against the existence of antiamboceptors.

In the main experiment cited by Pfeiffer and Moreschi, the results do not necessarily show the absence of antiamboceptors, but it does show the antibacteriolytic action produced by the action of complement and precipitate. Pfeiffer and Moreschi used an antiserum derived from rabbits by immunization with human serum. When human cholera serum was used as amboceptor, the serum precipitates and the supernatant fluids, they found that the precipitates exerted an antibacteriolytic action, while the supernatant fluid had no such action. From the conception of antiamboceptors furnished by Bordet's experiments, it might very well be assumed that antiamboceptors contained in the antiserum had been produced by the amboceptors present in the normal human serum. So far as the specific cholera amboceptors are concerned, these amboceptors accordingly have acted as "antibacteriolytic factors," and being so combined, their action as amboceptors is excluded. All that can be claimed for this experiment, is that it demonstrates the antibacteriolytic action of the

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er and Moreschi, Berliner klin. Wochenschr. No. 2, 1906.  
er and Moreschi (?)—[Translator].

precipitate. It sheds no light on the possibility of antiamboceptors being present in the antiserum at the same time.

The solution of this problem is simplified if we succeed in excluding the action of the precipitate and so permit the supposed antiamboceptor to act by itself. This can be accomplished by anchoring the amboceptor to the cell and removing the normal serum constituents by centrifuging. From this point of view, one may even consider the problem as already solved. The experiments of Bordet, Ehrlich and Sachs, Muir and Browning, with hæmolytic amboceptors, and those of Shibayama and Toyoda<sup>1</sup> with bacteriolytic amboceptors all agree in showing that the antiamboceptor acts even when the cell, loaded with amboceptor, has been separated from free serum constituents. Nevertheless, in view of the small traces of albuminous substance which suffice, when combined with suitable antibody, to deflect complement, it might be objected that it is difficult to completely free the sedimented blood-cells from traces of adherent albuminous substances. This difficulty would appear considerable, especially if we incline to believe that the blood-cells have some absorbing action on the albuminous substances. Furthermore, the antiamboceptors sometimes do not act at once on the amboceptor anchored to the cell. Bordet, for example, was unable to produce the antiamboceptor action until he suspended the blood-cells in inactive serum. This, of course, diminishes the value of the demonstration, since it introduces a possible interference due to complementoids (Muir and Browning). Our own observations lead us to believe that the ability of antiamboceptor to unite with the amboceptor bound to the cell or with the free amboceptor is very variable.

In view of these objections we have therefore attempted to demonstrate the presence of antiamboceptors indirectly, by excluding the action of antiamboceptors while allowing antibody and albuminous substances to participate in the reaction. It would seem that the simplest way to attain this would be to employ, *as the source of amboceptor, a different species of animal than was used for producing the antiamboceptor.*

The antiserum used by us was obtained from a goat which had been immunized with rabbit serum.<sup>2</sup> The amboceptor, of course,

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<sup>1</sup> Shibayama and Toyoda, *Centralbl. f. Bact., Orig.* Vol. XL, 1906.

<sup>2</sup> The serum with which these animals were immunized was derived from rabbits which had been treated with ox blood. It therefore contained specific

had to be derived from a rabbit. In the present instance it was an inactivated serum obtained from a rabbit treated with ox blood. Guinea-pig serum was used as complement. In order to exclude the action of the antiamboceptor, a parallel experiment was made in which the amboceptor consisted of the inactivated serum of a goat immunized with ox blood, guinea-pig serum being used as complement. For the sake of simplicity we shall term the amboceptors respectively "rabbit amboceptor" and "goat amboceptor." The experiment is as follows:

Two series of test-tubes were prepared, decreasing amounts of the anti-serum being placed in each tube. The volume in each tube was always made up to 1.0 cc. with physiological salt solution. To the tubes in series A was then added 0.0015 cc. ( $1\frac{1}{2}$  solvent doses) of the rabbit amboceptor; while the tubes of series B received 0.015 cc. goat amboceptor ( $1\frac{1}{2}$  solvent doses) plus 0.0015 cc. normal inactive rabbit serum. Both series of tubes were kept at room temperature for three-quarters of an hour, after which 1 cc. of a 5% suspension of ox blood-cells was added to each tube. After incubation at 37° for one hour, the tubes were centrifuged, the sediments resuspended in physiological salt solution, and mixed with guinea-pig serum as complement. The amount of complement also equaled  $1\frac{1}{2}$  solvent doses, being 0.075 cc. in series A, and 0.05 cc. in series B. After this the tubes were kept at 37° for two hours, and then placed in the refrigerator over night. The result noted the next morning is shown in the following table:

TABLE I.

Amount of Antiserum. cc.	Degree of Hemolysis.	
	Series A.	Series B.
0.5	0	strong
0.25	0	complete
0.15	0	"
0.1	0	"
0.05	0	"
0.025	strong	"
0.015	"	"
0.01	complete	"
0.005	"	"
0	"	"

amboceptors for ox blood. So far as the production of antiamboceptors or of antibodies against the albuminous substances is concerned, this is immaterial. Controls made with the serum of a goat immunized with normal rabbit serum, yielded the same results. The quantity of the latter available was too small to suffice for all of the experiments here reported.

In order to analyze the result of this experiment, it will be advisable to first have a clear idea as to the constitution of the sediments in the two series previous to the addition of the complement. In series A the sediment consists of:

1. The blood-cells laden with amboceptor.
2. The antiamboceptor (if such is present in the antiserum) bound to the complementophile group of the amboceptor.
3. It may contain the precipitate formed by the combination of albuminous constituents of the rabbit serum with the antiserum.

In series B the sediment also contains blood-cells laden with amboceptor, but there is, of course, no antiamboceptor. The conditions for the formation of the precipitate, however, are exactly the same as in series A, for in both series the same quantity of normal albuminous constituents of rabbit serum are present.

In series B, if we disregard the slight inhibition with large doses of antiserum, we find that the blood cells in all the tubes have been completely dissolved. This can only mean that either the sediments contained no precipitate, or that the precipitate present was unable to exert its deflecting power on complement. It follows that *the marked inhibition of hæmolysis observed in series A must be ascribed to the action of antiamboceptors.*

Against this interpretation it might be objected that perhaps the sediments of series A also lack an antiamboceptor, and that the inhibition of hæmolysis is due to the deflection of complement by the precipitate. It would then be necessary to assume that the goat amboceptor possessed a stronger affinity for the complement than did the rabbit amboceptor, in consequence of which no deflection of complement was produced by the precipitates in series B. In order to meet this objection we have devised another experiment, making use of the rabbit amboceptor as before, and excluding the antiamboceptor while still maintaining the same favorable conditions for the formation of a precipitate. The experiment is made as follows:

Decreasing amounts of antiserum are mixed with 0.0015 cc. inactivated normal rabbit serum, and the mixtures kept at room temperature for forty-five minutes. To each tube is then added 1 cc. 5% ox blood, the mixtures kept at 37° for one hour, and then centrifuged. The sediments are mixed with 0.0015 cc. rabbit amboceptor plus 0.075 cc. guinea-pig serum. It will be seen that the experiment corresponds to that described in Table I, A, except that in place of the specific amboceptor, an equal volume of normal serum is mixed



with the antiserum, the rabbit amboceptor being added to the mixture only after the antiamboceptor has been removed. The result of this experiment is shown in the following table (Column A of Table I may be regarded as the control):

TABLE II.

Amount of Antiserum. cc.	Degree of Hæmolysis.
0.5	slight
0.25	almost complete
0.15	complete
0.1	"
0.05	"
0.025	"
0.015	"
0	"

It will be seen that with this modification, too, the antiserum, except in very large amounts, does not influence hæmolysis. There can be no doubt, therefore, that *the inhibiting factor of the antiserum under these conditions is practically only the antiamboceptor*. While these experiments positively demonstrate the existence of antiamboceptors in the antiserum, they leave untouched the question as to whether the antiserum may not at the same time contain antibodies for albuminous substances. Considering the manner in which the antiserum is produced, it is natural to assume that such antibodies are formed along with the antiamboceptors. All that we are interested in at the present time, however, is the possibility of these antibodies counterfeiting the existence of antiamboceptors. After the experiments just described, this seems out of the question.

It might be doubted whether the albumin content of the normal rabbit serum corresponds to that of the rabbit serum specific for ox blood. The immune serum might be much richer in albuminous substances. Although there seems little basis for such an assumption, we have thought it advisable to investigate the matter. In a further experiment, therefore, we used varying quantities of the normal rabbit serum with constant amounts of the antiserum. The experiments were carried out as follows:

Two series of tests are made:

(A) Each tube contains 0.15 cc. antiserum, plus 0.0015 cc. rabbit amboceptor, plus decreasing amounts inactive normal rabbit serum. After standing,

forty-five minutes at room temperature, the ox-blood suspension is added and the mixtures kept for one hour at 37°. After centrifuging, the sediments are resuspended in physiological salt solution, and mixed with 0.075 cc. guinea-pig serum.

(B) Each tube contains 0.15 cc. antiserum, plus decreasing amounts of inactive normal rabbit serum. After standing for forty-five minutes at room temperature, the ox-blood suspension is added and the mixtures kept for one hour at 37°. After centrifuging, the sediments are mixed with 0.0015 cc. rabbit amboceptor, kept at 37° for one hour, and again centrifuged. To these sediments are then added 0.075 cc. guinea-pig serum.

In this experiment each series again contains the same constituents in like amounts, the main difference between them consisting in the sequence in which the constituents are added. By having varied this, we are enabled to exclude, in series B, the action of the antiamboceptor. The result of the experiment is shown in Table III.

TABLE III.

Amount of Normal Inactive Rabbit Serum. cc.	Degree of Hemolysis.	
	Series A.	Series B.
0.1	complete	complete
0.05	"	
0.025	"	
0.015	"	
0.01	strong	
0.005	moderate	
0.0025	0	
0.0015	0	
0.001	0	
0	0	

It will be seen that *despite an increased amount of precipitable substance, the precipitate exerts no binding action on complement. In series A, on the other hand, the inhibiting action of the antiamboceptor is again very marked.* The experiment also shows that a relatively slight excess of the normal rabbit serum paralyzes the antiamboceptor action, a fact which finds a natural explanation in the interference of the normal amboceptors.

At first sight the results shown in series A seem somewhat similar to those obtained in experiments made to determine the amount of albuminous substance necessary to produce deflection of comple-

ment when combined with the corresponding antiserum. We know from the researches of Fleischmann and Michaelis,<sup>1</sup> as well as from those of Moreschi,<sup>2</sup> that an excess of the albuminous antigen inhibits the deflection of complement. The same phenomenon is observed in the precipitin reaction. From the control furnished by series B, it is apparent that deflection of complement plays no part in the antihæmolytic action noted in series A. It follows, therefore, that the inhibition of hæmolysis observed when large amounts of serum are employed, is to be regarded as an antagonistic action exerted by the normal serum on the antiamboceptor, and must be ascribed to the normal amboceptors present.

In spite of this we may assume that in both series the blood-cell sediments contain an admixture of albuminous precipitate, for it could easily be shown that the antiserum possessed precipitating properties. The serum, to be sure, was rather weak, especially so far as the intensity of precipitation was concerned. It is to be noted, however, that even with the greatest excess of rabbit serum occurring in our experiments, there was no failure of precipitate formation; in fact, this increased in proportion to the amount of precipitable substance employed. Granted then, that the blood-cell sediments contained albumin precipitates, two alternatives may be offered to explain the lack of deflecting power on complements. Thus, it is possible that, despite the formation of a precipitate, there are no antibodies which are able to bind complement, or, if present, none that enter into the reaction. On the other hand, and this is important so far as the amboceptor problem is concerned, it is to be noted that with the technique employed by us, conditions have been introduced which render occurrence of deflection difficult or impossible. In order to produce deflection of complement, one proceeds by first mixing the albuminous antigen, antiserum, and complement, and subsequently adding blood cells and amboceptor. In our experiments, on the contrary, the resulting sediment already contains: 1, blood-cells laden with amboceptor, and 2, the precipitate. The complement which is now added finds two alternative points of attachment, and it depends entirely on the relative affinity possessed by these as to where the complement will be bound. Had the complement been allowed to react with the precipitate alone, it would undoubtedly

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<sup>1</sup> Fleischmann and Michaelis, *Mediz. Klin.*, No. 1, 1906.

<sup>2</sup> Moreschi, *l. c.*

have been anchored, and then, owing to the secondary tightening of the bonds, would no longer have been available even for a group possessing somewhat higher affinity. One can easily convince one's self of the part played in deflection by the sequence in which the various reagents are added. In a recent study, Michaelis and Fleischmann<sup>1</sup> have called attention to the sources of error to which disregard of this circumstance may give rise.

We made the following experiment with our antiserum:

Two series of tubes were prepared, each containing 0.1 cc. antiserum and decreasing amounts of normal rabbit serum. The volume was made up to 1.5 cc. and the mixtures allowed to stand for twenty-four hours in order to secure the maximum amount of precipitation. The tubes were sharply centrifuged, and the supernatant fluid removed. To the sediments in series A were then added 0.075 cc. guinea-pig serum, and the mixtures kept at 37° for one hour. Then the ox blood, plus 0.0015 cc. rabbit amboceptor, was added. In series B, the sequence was altered to: ox blood, plus 0.0015 cc. amboceptor—one hour at 37°—then 0.75 cc. guinea-pig serum.

The degree of hæmolysis at the end of 1½ hours is shown in Table IV.

TABLE IV.

Amount of Normal Rabbit Serum. cc.	Degree of Hæmolysis.	
	Series A.	Series B.
0.25	0	complete
0.15	faint trace	"
0.1	"	"
0.05	strong	"
0.025	"	"
0.015	"	"
0.01	almost complete	"
0	complete	"

It will be seen that the precipitate has exerted a deflection of complement, though not to a very high degree; there is no deflection, however, when the sequence in which the various reagents are added is the same as that employed in our antiamboceptor experiments.<sup>2</sup>

The essential importance of the technique employed, when

<sup>1</sup> Michaelis and Fleischmann, Zeitsch. f. klin. Medizin. Vol. 58, 1906.

<sup>2</sup> It is impossible for us to say whether the sequences in which the reagents are added would have the same determining influence when other amboceptors, especially bactericidal amboceptors, are employed.

making experiments on the deflection of complement, was also well demonstrated by using a strong precipitating serum which had previously been employed for identifying human albumin. This serum was obtained from rabbits by immunization with human serum, and is therefore termed "H-R-serum." Since the only antiamboceptors which this serum can contain are those directed against human amboceptors, it is obvious that an antiamboceptor action is at once excluded if we employ rabbit amboceptors specific for ox blood. We began by following the regular technique employed by M. Neisser and Sachs<sup>1</sup> in their studies on the forensic blood test by means of antihæmolytic action, and followed this by two parallel experiments in which we varied the sequence of the reagents employed. The details of the three tests are as follows:

*Series A.* Each tube contains 0.02 cc. H-R-serum, plus 0.05 cc. guinea-pig serum, plus decreasing amounts human serum. Mixtures kept one hour at 37°. Then 1 cc. 5% ox blood, plus 0.0015 rabbit amboceptor.

*Series B.* 0.02 cc. H-R-serum, plus human serum, plus 1 cc. 5% ox blood, plus 0.0015 rabbit amboceptor. After standing one hour at 37°, 0.05 cc. guinea-pig serum.

*Series C.* 0.02 cc. H-R-serum, plus human serum, plus 1 cc. 5% ox blood, plus 0.0015 rabbit amboceptor. After standing for 1½ hours at 37°, the mixtures are centrifuged. Then 0.05 guinea-pig serum is added to the sediments.

In series C, the mixtures were kept at 37° for 1½ hours in order to furnish more favorable conditions for the formation of a precipitate, and also so that the conditions as to time would be the same as those in the antiamboceptor experiment. In series B and C, the guinea-pig serum was kept at 37° for one hour previous to mixing.

The result of this experiment is shown in Table V.

TABLE V.

Amount of Human Serum. cc.	Degree of Hæmolysis.		
	Series A.	Series B.	Series C.
0.001	0	trace	complete
0.0001	0	"	
0.00001	0	moderate	
0.000001	0	complete	
0	complete	"	

<sup>1</sup> Neisser and Sachs, Berliner klin. Wochenschrift, No. 3, 1906.

In series A we see deflection of complement very well marked; in series B, in which complement was added last, the deflection is considerably lessened, and when the additions are made in accordance with the technique of the antiamboceptor experiment, we find that there is no deflection whatever. In this experiment, as already explained, we made use of an antiserum having strong precipitating and deflecting power. The result confirms our contention that the inhibiting action observed in our previous experiments is not due to the formation of a precipitate, but is caused solely by anti-amboceptors.

Even when present, the precipitates are unable to exert a deflection on the complement provided blood-cells laden with amboceptor are present at the same time, so that the complement subsequently introduced has the alternative of combining with precipitate or with the prepared blood-cells. At the same time we must call attention to a possibility which makes it likely that an intensification of the power of the precipitate occurs in connection with the antiamboceptor action. Conditions might exist under which the complement would replace the antiamboceptor already bound to the amboceptor, were not the precipitate present at the same time. It is possible that this explains the varying results obtained in attempts to definitely replace with antiamboceptor the normal amboceptor already anchored to the cell, and freed from normal serum constituents.<sup>1</sup> It is con-

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<sup>1</sup> Ehrlich and Sachs (l.c.) called attention to a paradoxical phenomenon, which consisted in the fact that the sensitized blood-cells were protected only by small doses of the antiserum, while an excess of antiserum did not inhibit hæmolysis. They found, however, that the anti-hæmolytic effect was produced even with an excess of antiserum, provided a small quantity of normal serum homologous to the amboceptor was added. Moreschi (l.c.) interprets this as indicating an anticomplementary action due to the formation of a precipitate. In opposition to this, it may be remarked that under analogous conditions the formation of a precipitate does not lead to a deflection of complement. The peculiarity of the phenomenon described by Ehrlich and Sachs consists not alone in the fact that the antiserum acts only after the addition of normal serum. The striking thing is that an excess should cause the antiserum to lose its inhibiting property. In this the presence and coaction of normal serum constituents (precipitable substances) are entirely out of the question. Hence, while at first sight Moreschi's explanation appears very apt, we see that it is insufficient to throw light on the entire group of facts presented by Ehrlich and Sachs. For the present it will be difficult to get along without accepting the possibility suggested by those authors, namely,

ceivable that even under these circumstances, the antiamboceptor is always bound, but that in many cases this union can still be dissolved by the complement owing to the absence of the deflecting precipitate. If we accept this *secondary* participation of the precipitate in the antiamboceptor action, it is easy to understand the apparent failure of the antiamboceptor to be bound to the sensitized blood-cells. Some explanation for this lack of combination is certainly desirable. One would naturally expect the antiamboceptor to act more powerfully on the sensitized blood-cells, for in blood-cells laden with amboceptor the free, normal amboceptors of the immune serum are absent. These free amboceptors come into action when the antiamboceptor acts directly on the entire immune serum, and they can thus lower the action of the antiamboceptor on the specific amboceptor. As a matter of fact, we have encountered instances in which the antiserum acted just as strongly on the sensitized cell as on the native immune serum. In other cases, however, the antiserum, when employed in accordance with the usual technique (sensitized blood+antiserum—one hour at 37°—centrifuging—addition of complement—two hours at 37°), exerted no action whatever. This was the case with the antiserum whose properties we have discussed in this paper.

These considerations led us to see if we could make the action of the antiserum on the sensitized cell visible. To do this we felt that two things in particular had to be regarded. In the first place, it seemed advisable to leave the antiserum in contact with the blood-cells laden with amboceptor as long as possible, in order to effect the maximum amount of binding with the antiamboceptor. This would make it more difficult for the complement subsequently added to dislodge the antiamboceptor. In the second place, it seemed probable that the complement only gradually displaced the antiamboceptor, and that examinations made at intervals would reveal a phase in which an antiamboceptor action can be observed.

We arranged our experiment as follows:

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an interfering action produced by two antibodies in the antiserum, bodies having the type of antiamboceptors. So far as the details are concerned we must refer to the original paper of Ehrlich and Sachs. Here we would only remark that the interpretation given at that time is applicable also to those cases in which the antiamboceptor is without effect when sensitized blood-cells freed from normal serum constituents are employed.

To 1 cc. 5% ox blood are added 0.0015 cc. rabbit amboceptor, and the mixtures kept at 37° for one hour. Each tube receives decreasing amounts of antiserum, those in series A directly, and those in series B, to the blood-cells separated by centrifuge and freed from the fluid medium in which they had been suspended. The two series therefore contained, in addition to the antiserum:

*Series A.* Blood-cells laden with amboceptor, plus free normal amboceptors, plus precipitable substance.

*Series B.* Only blood-cells laden with amboceptor.

Both sets of tubes are kept at 37° for two hours, then in the refrigerator over night, and centrifuged the next morning. The sediments are suspended in physiological salt solution to which, for each tube, 1½ solvent doses of guinea-pig serum have been added (0.03 cc.). The degree of hæmolysis is noted at the end of ½ and 2 hours. See Table VI.

TABLE VI.

Amount of Antiserum. cc.	Degree of Hæmolysis.			
	Series A.		Series B.	
	After ½ Hour.	After 2 Hours.	After ½ Hour.	After 2 Hours.
0.25	0	0	0	moderate
0.15	0	0	0	"
0.1	0	faint trace	0	"
0.05	faint trace	strong	0	strong
0.025	trace	complete	0	"
0.015	moderate	"	0	almost complete
0.01	almost complete	"	moderate	complete
0.005	"	"	"	"
0.0025	complete	"	"	"
0.0015	"	"	strong	"
0.001	"	"	"	"
0	"	"	complete	"

A number of points are brought out by this table. In series B we observe that the antiamboceptor has exerted a distinct influence on the antiamboceptor<sup>1</sup> anchored by the cells and freed from other serum constituents. Examining the tubes at the end of half an hour we see that hæmolysis has been markedly inhibited. Subsequently, however, this inhibition gradually disappears, so that at the end of two hours what little antihæmolytic action is still present is insignificant when compared to the antiamboceptor action at the end of half an hour. This result agrees very well with the assumption that the complement is able, after a time, to dislodge the antiamboceptor. On comparing the results in series B with those in series A, we note that the

<sup>1</sup> Misprint for amboceptor (?)—[Editor.]



inhibition of hæmolysis at the end of half an hour is less marked in the latter. One would have expected the contrary to be the case, or the presence of precipitable substance in series A furnishes conditions favorable to the formation of a precipitate. It must not be forgotten, however, that the mixtures in series A also contain free normal amboceptors (eliminated in series B) and these may be able to diminish the antiamboceptor action. This is all the more likely since these amboceptors are free in solution and therefore more readily able to react with the antiamboceptors than are the specific amboceptors already bound to the cell.

At the end of two hours, on the other hand, we find that the antiamboceptor action is more marked in series A than in series B. On the basis of the above assumption, this might be due to the fact that the precipitate produced by the large quantities of antiserum is, in a way, a deflector of complement, since it robs the complement of its tendency to break up the amboceptor-antiamboceptor combination. Under the conditions obtaining, the complement-binding power of the precipitate is too small to prevent the complement uniting with the free complementophile group of the amboceptor, but is large enough to restrain it when the complementophile group is already occupied by the antiamboceptor. *Precipitate and antiamboceptor would thus at times mutually support each other in their action.*

To what extent such a combined action really occurs must be left to future investigations. In any case, we believe it important to bear this possibility in mind, in order to gain a clear idea of all the conditions which may play a part in the action of anti-amboceptors.

Each of the two factors (precipitate and antiamboceptor) will surely also be able to exert an antihæmolytic effect by itself. The independent action of the antiamboceptor is demonstrated further by the fact that it persists even when the complement is increased several times. If the inhibition were due *only* to precipitates, we should expect that it would be overcome by an excess of complement, since the precipitate acts only as an anticomplement. On the contrary, it can be shown that the inhibition produced by the anti-amboceptor persists even when the dose of complement is considerably increased. It might be thought that a precipitate present at the same time binds all the complement added, but this is not the case. It is possible to demonstrate the presence of sufficient free complement by separating the fluid from the undissolved blood-cells, and allowing it to act on native, sensitized blood-cells. This fact agrees with Bordet's observation, that the antiamboceptor robs sensitized blood-cells of the power to bind complement. When we employed a very small quantity of complement, just sufficient to produce com-

plete hæmolysis, we did, to be sure, observe a slight loss of complement, despite the presence of antiamboceptor. We believe that this is caused by the presence of a very small amount of precipitate. The important fact, however, is that we could demonstrate plenty of free complement, although there was no hæmolysis.<sup>1</sup> A brief description of such an experiment follows:

To 0.125 cc. antiserum are added 0.0015 cc. rabbit amboceptor, and the mixtures kept at room temperature for 45 minutes. Ox blood is added, and the mixtures kept at 37° for one hour. After centrifuging, the sediments are mixed with guinea-pig serum, as follows:

1. 0.075 cc. = 1½ complete solvent doses.
2. 0.1 cc. = 2 solvent doses.
3. 0.2 cc. = 4 solvent doses.
4. 0.3 cc. = 6 solvent doses.

The tubes are kept at 37° for two hours, then over night in the ice chest. The following day the supernatant fluids are carefully poured off and tested for complement by adding the sediments obtained from 1 cc. 5% ox blood plus 0.0015 cc. rabbit amboceptor. The result is shown in Table VII.

TABLE VII.

Amount of Guinea-pig Serum. cc.	Degree of Hæmolysis.	
	(a) Of the Original Mixtures.	(b) Of the Decanted Fluids Digested with Ox Blood plus Amboceptor.
0.075	0	strong
0.1	0	complete
0.2	0	"
0.3	0	"

Although as indicated in the first column of the table, there is a moderate diminution of complement, we note that despite a plentiful amount of complement, hæmolysis does not occur. The reason for

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<sup>1</sup> We see, therefore, that the ability of the complement to dislodge anchored antiamboceptor (if such a power is at all possessed by the complement) does not always manifest itself.

this is because the complementophile group of the amboceptor is occupied by the antiamboceptor, whereby this point of attachment is blocked for the complement as with a complementoid.

Summing up the results of our experiments, we must conclude that it is impossible longer to doubt the existence, in the antiserum, of antibodies directed against hæmolytic amboceptors. It is possible to differentiate them in their action, even when antibodies for albuminous substances are present at the same time. This establishes the antiamboceptors as inhibiting substances *sui generis*. By the formation of precipitates, the albumin-antibodies may, at times, more or less favor the action of the antiamboceptors, without, however, exhibiting the complement-binding power inherent in them.

## XLVIII. DISSOCIATION PHENOMENA IN THE TOXIN-ANTITOXIN COMBINATION.<sup>1</sup>

By Doctors R. OTTO and H. SACHS.

IN recent years a number of investigators have called attention to a curious paradoxical phenomenon, namely, that with suitable mixtures of toxin and antitoxin the toxicity for animals is the greater up to a certain point, the smaller the fractional part injected. It is to the keen observation of Behring<sup>2</sup> that we owe the first data on this subject. Behring found that the injection of 1-50, or even 1-500, part of a mixture of tetanus toxin and tetanus antitoxin was more highly toxic for mice than the injection of the entire amount. It should at once be stated that the fractional parts were diluted with water, so that the volume injected was the same in all cases. Analogous observations were recently made by Madsen<sup>3</sup> working with the toxin of botulism. This investigator found that toxin-antitoxin mixtures which exerted only very slight toxic effects might still kill guinea-pigs, if but the fortieth or eightieth part of the mixture were used. Similarly, the slight toxic effects of the full amount could be entirely avoided if ten times this quantity was injected. We, too, encountered the phenomenon some years ago in the course of test tube experiments on the hæmolytic action of garden-spider toxin. After the publication of Madsen's observations, we took up the question anew, and studied the phenomenon in mixtures of botulism toxin and antitoxin. In view of the interest which attaches to the

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<sup>1</sup> Reprinted from *Zeitschr. f. exp. Pathol. u. Therapie*, Vol. III, 1906.

<sup>2</sup> E. von Behring, *Aetiologie und aetiologische Therapie des Tetanus*. Behring's Beiträge zur experimentellen Therapie, Heft 7, 1904, p. 51; also *ibid.* Heft 3, 1900, p. 1092.

<sup>3</sup> Th. Madsen, *Gifte und Gegengifte*, *Centralblatt f. Bacteriologie, Referate*, Vol. 37, 1905; also *Proceedings of the Danish Academy of Sciences, Meeting*, Dec. 16, 1904.

subject, we have felt it advisable to publish the results of our experiments, especially since they shed some light on the cause of the paradoxical results.

The botulism toxin and its antitoxin was kindly furnished us by Professor Forssmann of Lund. We began by experimenting with mice, and first determined the lethal dose by subcutaneous injections. This is shown in the following table:

TABLE I.

Dose of Toxin.	Effect on the Animal.	Remarks.
0.0002	†2*	—
0.0001	†3	—
0.00009	lives	sick 8 days
0.00008	"	sick 3 days

\* †2, etc., denote death on the second day, etc.

We next determined the  $L^+$  quantity of the antitoxin, using 1,000 times the fatal dose (0.1 cc.) for this purpose. The mixtures of toxin-antitoxin were allowed to stand for three hours at room temperature previous to injection. The result of this test is shown in Table II.

TABLE II.

Dose of Toxin.	Dose of Antitoxin.	Effect on the Animals.	Remarks.
0.1	0.001	lives	—
0.1	0.0009	"	—
0.1	0.0008	"	—
0.1	0.0007	"	sick 1 day
0.1	0.0006	"	sick 4 days
0.1	0.0005	†4	—
0.1	0.0004	†2	—
0.1	0.0003	†1	—

The experiments proper began with a mixture of toxin, 0.1 + antitoxin, 0.0006. The mice were injected subcutaneously with 1/1, 1/2, 1/5, 1/10, etc., of this mixture. The dilutions were prepared immediately before the injection, and the volume of the fluid injected was always 1 cc. The result is shown in Table III.

TABLE III.

Fractional Part of the Mixture (0.1 Toxin + 0.0006 Anti- toxin) Injected.	The Injection was Made			
	A Directly after Mixing.		B After Three Hours' Standing.	
	Effect.	Remarks.	Effect.	Remarks.
1/1	lives	sick 2 days	lives	sick 3 days
1/2	"	sick 4 days	"	sick 3 days
1/5	†2	—	"	sick 5 days
1/10	†1½	—	†3	—
1/20	†2	—	†3	—
1/50	†2	—	†5	—
1/75	†2	—	†4	—
1/100	†3	—	lives	sick 2 days

The table needs no further explanation. It completely confirms the results obtained by Madsen, and exhibits the paradoxical phenomenon in the clearest manner. It should be noted that it makes very little difference whether the dilutions of the original mixture and the injections are made immediately after preparing the mixture or after the latter has stood for three hours, though the phenomenon is perhaps somewhat more striking if the injections are made at once.

A deeper insight was afforded when we used rabbits for the inoculations, for then we were able to apply the toxin-antitoxin mixtures by means of intravenous injections. A comparison of the  $L^1$  values in rabbits, both with subcutaneous and intravenous injections, at once showed marked differences. Thus when we injected toxin-antitoxin mixtures which had stood three hours, we found the intravenous injections to be considerably more toxic than the subcutaneous. If, however, we waited 24 hours after preparing the mixtures, and then injected, we found that this difference was practically wiped out. Such an experiment is reproduced in Table IV.

From the table we see that the toxicity of the mixtures by subcutaneous injection has been but slightly altered by the 24 hours' standing; there is perhaps a little impairment, but it is inconsiderable. When intravenous injections are employed, however, a marked loss of toxicity is caused by the twenty-four hours' standing. In the case of this botulism toxin we are apparently dealing with the same conditions which Morgenroth has described in the case of diphtheria

TABLE IV.

(A) DETERMINATION OF  $L_{\dagger}$  WHEN MIXTURES HAVE STOOD 3 HOURS.

Dose of Toxin.	Dose of Antitoxin.	Subcutaneous.		Intravaneous.	
		Effect.	Remarks.	Effect.	Remarks.
0.1	0.0004	†4	—	†2	—
0.1	0.0007	lives	sick 2 days	†3	—
0.1	0.001	—	—	†3	—

(B) DETERMINATION OF  $L_{\dagger}$  WHEN MIXTURES HAVE STOOD 24 HOURS.

0.1	0.0001	†3	—	†2	—
0.1	0.0002	†4	—	†4	—
0.1	0.0004	†17	—	†16	—
0.1	0.0007	lives	lively	lives	slightly ill 3 days lively
0.1	0.001	—	—	lives	

toxin. Morgenroth<sup>1</sup> found that the reaction between diphtheria toxin and its antitoxin proceeded slowly, but that the time relations could be brought out only by means of intravenous injections. When subcutaneous injections were employed, the length of time which the toxin-antitoxin mixtures remained in contact appeared to have no influence whatever. Morgenroth therefore assumed "that in the subcutaneous areolar tissue certain factors are present which hasten the union of toxin and antitoxin." His idea, then, is that the reaction is hastened by certain positive catalytic influences.<sup>2</sup> We shall probably not err if we interpret our own results, with botulism toxin, in the same manner, and assume that they are the result of a slow reaction between toxin and antitoxin, which reaction is hastened in the subcutaneous connective tissue.

In view of these facts one might assume that the increased toxicity of fractional portions of a relatively neutral toxin-antitoxin mixture was due to the catalytic action of the tissues, somewhat in

<sup>1</sup> Morgenroth, Untersuchungen über die Bindung von Diphtherietoxin und Antitoxin, zugleich ein Beitrag zur Kenntniss der Constitution des Diphtheriegiftes. Zeitschrift f. Hygiene, Vol. XLVIII, 1904; also Berliner klin. Wochenschr., No. 20, 1904.

<sup>2</sup> Attention may be called to the fact that von Behring assumed the existence of a positive katalysator (conductor) in fresh tetanus antitoxin. See Deutsche med. Wochenschrift, No. 35, 1903.

the following manner: the original mixture injected subcutaneously is not yet completely neutralized and becomes so only through the catalytic action of the subcutaneous tissues. It is conceivable that this catalytic action might become less with decreasing concentration of the toxin-antitoxin mixture, so that the original concentrated solution proved non-poisonous while a fractional part of the same, through the absence of the neutralizing catalytic action, would still be toxic. Such an assumption would at least explain certain of the observed facts. It seemed advisable, therefore, to repeat the experiments in such a way as to exclude the catalytic action of the subcutaneous tissue and this was easily possible by injecting rabbits intravenously. The determinations of the  $L^1$  dose for rabbits are shown in the following table:

TABLE V.

Amount of Toxin.	Amount of Antitoxin.	Intravenous Injection after			
		Standing 3 Hours.		Standing 24 Hours.	
		Result.	Remarks.	Result.	Remarks.
0.5	0.001	—	—	†2	—
0.5	0.0015	—	—	†3	—
0.5	0.002	†2	—	†6	—
0.5	0.003	†4	—	lives	ill 2 days
0.5	0.004	†15	—	—	—
0.5	0.005	lives	lively	—	—

Having obtained these data, we injected two series of rabbits with dilutions of the following mixtures:

- (a) 0.5 toxin plus 0.004 antitoxin standing 3 hours.
- (b) 0.5 toxin plus 0.003 antitoxin standing 24 hours.

The result of the experiment is shown in Table VI.

From this table we see at once that even when intravenous injections are employed, the increased toxicity of fractional portions of toxin-antitoxin mixtures is still strikingly manifested. We shall, therefore, have to assume that really neutralized mixtures of toxin and antitoxin become more toxic on dilution, that, in other words, there is a dissociation of the toxin-antitoxin combination when the mixtures are diluted. From Table VIb, moreover, we learn that this dissociability almost disappears when the mixtures have stood



TABLE VI.

Fractional Portion of the Mixture Injected.	Mixture a.		Mixture b.	
	Result.	Remarks.	Result.	Remarks.
1/1	lives	ill 2 days	lives	well(?)
1/2	†5	-	"	"
1/4	†5	—	"	ill a long time
1/8	lives	ill 4 days	"	well(?)
1/16	lives	well	"	ill several days
1/32	lives	"	"	well

for some time. With mixtures that have stood 24 hours before diluting, there is practically no increase in toxicity as a result of dilution, and this is all the more noticeable because the mixture which stood 24 hours contained only three-quarters of the quantity of toxin contained in that which stood only three hours. It is necessary, therefore, to distinguish two phases in the reaction between toxin and antitoxin, a primary phase in which neutralization has taken place, but in which dilution suffices to again liberate some of the toxin, and a secondary phase in which this is no longer possible or is possible only to a very slight degree. The assumption of these two phases accords completely with Ehrlich's views concerning the relations existing between toxin and antitoxin. We assume that in the toxin-antitoxin reaction there exists a stage in which the reaction is to a certain extent reversible, and that this is succeeded by a tightening of the bonds, a stage of firm union, in which the reversibility is lost. The most striking example of this secondary tightening is that known as the Danysz-Dungern<sup>1</sup> phenomenon, which consists in the demonstration of increased toxicity of toxin-antitoxin mixtures by the fractional additional of the toxin.

In the phenomenon which we are studying, the first stage of the reaction, namely, that of reversibility, is brought out by diluting the mixtures. It has, of course, long been known that the union of toxin and antitoxin proceeds more rapidly in concentrated than in dilute solutions, and this has from the outset been emphasized by Ehrlich. What was new about these observations was the fact that neutralized, concentrated toxin-antitoxin mixtures could be disso-

<sup>1</sup> v. Dungern, *Deutsch. med. Wochenschr.*, 1904; Sachs, *Berl. klin. Wochenschr.*, 1904; and *Centralbl. Bacteriol. I Abt., Orig. Vol. XXXVII*, 1904.

ciated to so great a degree by diluting the mixtures. The process reminds one in a way of the well-known chemical phenomenon of hydrolytic dissociation. To mention but a single example, acetic acid and alcohol unite to form ethylacetate. Conversely, however, when diluted with water, ethylacetate decomposes into its two components, acetic acid and alcohol. If we regard the toxin as the acetic acid, the antitoxin as the alcohol, and the neutral toxin-antitoxin mixture as the product of the two, ethylacetate, we get a good picture of what occurs when we dilute the toxin-antitoxin mixture. Our experiments show, then, that by diluting neutral toxin-antitoxin mixtures it is possible to recover the two components, toxin and antitoxin, up to a certain point. Furthermore, the possibility of doing this by dilution exists for only a comparatively short time. After this the secondary tightening of the bonds effects such a firm union that this mode of separating the two components does not avail. By making use of special methods, however, it is possible, even after a considerable time, to liberate the toxin from a neutral toxin-antitoxin mixture. This is well shown by the interesting experiments recently published by Morgenroth.<sup>1</sup> This author showed that by allowing hydrochloric acid to act on a neutral mixture of cobra venom and its antitoxin, complete dissociation could be effected, so that the entire amount of the two substances could be recovered. Morgenroth rightly regards this demonstration as an important argument in favor of the chemical theory of the toxin-antitoxin reaction, and emphasizes the fact that this behavior in no way contradicts the stereochemical conception formulated by Ehrlich.

Conditions apparently are such that after the union has become firm only the intense influence of powerful agents, such as hydrochloric acid in the case before us, or ferments in the case of glucosides, are able to effect dissociation. In contrast to this, we see that the dilution phenomenon studied by us is demonstrable only during the stage of loose union, mere dilution being unable to effect dissociation after the union has become firm. It is evident, from what has been said, that it is impossible to analyze these reactions according to the principle of the Guldberg-Waage law. Objection must also be made to the attempts to view these relations from the standpoint of colloid chemistry. These attempts grow out of purely external

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<sup>1</sup> Morgenroth, Ueber die Wiedergewinnung von Toxin aus seiner Antitoxin-  
verbindung, Berliner klinische Wochenschrift, 1905, No. 50.

analogies which in no way warrant abandoning the structuro-chemical conception. The latter alone has been able to do justice to the manifold phenomena under discussion.

We have seen that the increased toxicity effected by dilution is not dependent on any special vital influences on the part of the animal injected. This point is still further confirmed by experiments which we made with arachnolysin (the hæmolytic principle of the garden spider <sup>1</sup>), in which we were able to reproduce the same conditions in test-tube experiments.<sup>2</sup>

The serum employed in our experiments was obtained by immunizing rabbits against arachnolysin. This poison is particularly well suited for experiments of this kind because it is very resistant and because the reaction between arachnolysin and antilysin is practically completed in an hour. During the first hour, to be sure, the course of the reaction is a gradual one. The blood used was always 1 cc. of a 5% suspension of rabbit blood. Of the arachnolysin 0.2 cc. (approximately 200 complete solvent doses) were mixed with varying amounts of antilysin and the mixtures made up to an equal volume (8 cc.) with physiological salt solution. The first titration of the mixture was undertaken at the end of an hour, and a second at the end of 24 hours. The contents of each tube was always made up to 2 cc. with salt solution. The result of the experiment is shown in Table VII.

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<sup>1</sup> Sachs, Zur Kenntniss des Kreuzspinnengiftes. See this volume, page 167.

<sup>2</sup> Madsen, to be sure, mentions similar observations in the case of saponin and cholesterin. His experiments, however, do not impress us as justifying the analogizing conclusion which he draws. Thus one sees that the determinations of the hæmolytic power of the saponin do not proceed quite regularly; the saponin by itself, in his tests, sometimes acts more powerfully in small doses than in large. Then, too, in the titrations of the saponin-cholesterin mixtures there are zones of marked action from which there is diminished hæmolysis both with larger and with smaller doses. Finally, it should be noted that this diminution is succeeded upwards by a progressive increase of hæmolysis, reaching its maximum with the largest dose of the mixture. It is evident, therefore, that these experiments of Madsen have nothing to do with the phenomena observed by him or with those observed by us with the toxin of botulism. We are unable to say what causes the irregularities in the saponin-cholesterin tests. The mechanism of the action of cholesterin on saponin is manifestly entirely different from that of the toxin-antitoxin reaction.

TABLE VII.

## A.

THE HÆMOLYTIC POWER DETERMINED AT THE END OF 1 HOUR.

Amount of Mixture. cc.	The Mixture was Composed of 0.2 cc. Arachnolysin + Antiarachnolysin,				
	2.4 cc.	2.0 cc.	1.6 cc.	1.2 cc.	0.8 cc.
1.0	0	0	faint trace	moderate	complete
0.5	0	0	trace	"	"
0.25	0	faint trace	moderate	marked	"
0.15	0	trace	complete	"	"
0.1	0	"	moderate	moderate	"
0.05	faint trace	slight	"	"	"
0.025	"	trace	slight	slight	marked
0.015	"	"	trace	"	"
0.1	"	faint trace	faint trace	"	moderate

## B.

THE HÆMOLYTIC POWER DETERMINED AT THE END OF 24 HOURS.

1.0	}	0	}	0	faint trace	moderate	complete
0.5					trace	"	"
0.25					"	marked	"
0.15					"	"	"
0.1					"	moderate	"
0.05					"	"	"
0.025					faint trace	"	marked
0.015					"	slight	"
0.01					"	trace	"

The table shows that in relatively fresh mixtures of arachnolysin and antiarachnolysin the dilution phenomenon can be strikingly demonstrated. With the mixtures which have stood for 24 hours, however, the power of acting more strongly in smaller doses has largely disappeared, though even here there is some indication of the curious phenomenon. In fact, even after 48 and 72 hours the phenomenon is present to a slight degree. We see, therefore, that the results with arachnolysin correspond entirely with those observed with botulism toxin, and the same explanation applies.

Before closing this paper, we must call attention to a remarkable observation made in the course of these experiments. On resuming, this summer, the work which we had begun a year and more before, we found it impossible to reproduce the paradoxical phenomenon with the old sera left from the original experiments. We therefore

prepared fresh antilytic sera by immunizing rabbits, and found that these at once gave the paradoxical results under discussion. Concerning the cause of this peculiar behavior of fresh and old antitoxin we can only offer conjectures. One could assume that on standing the antitoxin becomes changed into a form possessing greater affinity. It must be admitted that the experiences had with other sera, both antitoxic and bactericidal, do not lend support to this assumption, since thus far we know age merely to weaken the sera but not to increase the antitoxic action. It is more natural, therefore, to assume that the serum contains substances which act like negative catalyzers. Thus, while the positive catalyzers already mentioned hasten the toxin-antitoxin reaction, the negative catalyzers assumed to exist in the serum would retard the tightening of the union. One would then say that the fresh antiarachnolysin serum contained the negative catalyzer, and that this by retarding the tightening of the union, made possible the dissociation of the two components when the mixtures were diluted. In an old serum, on the other hand, this retarding substance would be absent, thus making the toxin-antitoxin union firm in a very short time. In that case, of course, the dilution phenomenon could not be demonstrated.

We believe that a mere study of the successive events in the toxin-antitoxin neutralization permits of no direct conclusions. We have seen that it is impossible to exclude certain factors which markedly affect the course of the reaction; the existence of positive catalyzers had necessarily to be assumed, and the influence of negative catalyzers was rendered probable by the results of our investigations. It is therefore impossible by a mere numerical analysis of the course of the experiment to draw definite conclusions concerning the absolute combining affinity in the toxin-antitoxin reaction.

## XLIX. THE PARTIAL-FUNCTIONS OF CELLS.<sup>1</sup>

By Prof. PAUL EHRLICH.

THE history of our knowledge of vital phenomena and of the organic world can be divided into two parts. For a long time anatomy, especially the anatomy of the human body, constituted the beginning and the end of scientific knowledge. Further progress was only made possible by the invention of the microscope. Many years, however, passed by before Schwann demonstrated the cell as the final biologic unit. It would be like carrying wisdom to Athens to sketch for you the immeasurable progress which we owe to the introduction of the cell concept, the concept about which the entire modern science of life turns.

I take it to be generally accepted that everything which goes on within the body, assimilation and disassimilation, is referable, in the final analysis, to the cell; that the cells of different organs are differentiated from each other in a specific manner, and that this differentiation makes it possible for them to fulfill their various functions.

The results mentioned were achieved principally by histological examinations of dead and living tissues, though the allied sciences, physiology, toxicology, and especially comparative anatomy and biology, made most valuable contributions. Nevertheless I am inclined to believe that the aid which the microscope has given and can still give us is approaching a limit, and that in a deeper analysis of the all-important problem of cell life the application of optical contrivances, no matter how delicate, will fail us. The time has come for a further study of the minute chemistry of cell life; the concept *cell* must be resolved into a large number of distinct *partic*

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<sup>1</sup> The Nobel Lecture, delivered in Stockholm, Dec. 11, 1908. Reprinted from *Münchener mediz. Wochenschrift*, No. 5, 1909.

*functions.* The activities of a cell, however, are essentially chemical in nature, and since the formation of chemical structure is beyond the pale of visibility, it follows that we must cast about for other methods of study. This is important not only for a real understanding of vital phenomena, but because it constitutes the basis of a truly rational use of drugs.

The first step in this complicated domain was taken, as is often the case, quite indirectly. Following Behring's great discovery of the antitoxins, I sought to gain a deeper insight into the nature of their action, and after considerable study succeeded in finding the key to the mystery.

You all know that the power to excite the production of antibodies is confined to a distinct group of poisonous substances, the so-called toxins. These are products of the metabolism of animal or vegetable cells: diphtheria and tetanus toxins, abrin, ricin, snake venom, and many others. None of these substances can be crystallized; all seem to belong to the class of substances spoken of as albuminoid. In general the toxin is characterized by two properties, first, its toxicity, second, its power to excite the production of a specific antitoxin in the animal body.

In my quantitative investigations concerning this process I found that the toxins, especially solutions of diphtheria toxin, underwent a peculiar transformation, either spontaneously on standing, or through the action of thermic or chemic influences. While their toxicity was lost to a greater or less extent, their power to excite antibody production in the animal body remained intact. Furthermore, it was found that these transformation products, which I term toxoids and which my esteemed friend, Professor Arrhenius, has encountered in his numerous experiments, these toxoids still retained the power to specifically neutralize the antitoxin. In fact, in favorable cases it was possible to demonstrate that the transformation of toxin into toxoid is quantitative, i. e., a certain poison solution would neutralize exactly the same amount of antitoxin before as after the transformation into toxoid.

These facts permit of but one explanation, namely, that the toxin possesses two groups having different functions. One of these which remains intact in the "toxoid" and which therefore is to be regarded as the more stable, must possess the property of exciting the production of antibodies when injected into an animal, and must also be able to neutralize the antibody both in a test tube and in

vivo. Since, however, the relations existing between toxin and its antitoxin are strictly specific (tetanus antitoxin neutralizes only tetanus poison, diphtheria serum only diphtheria poison, snake antivenin only snake venom, etc., etc.) it is necessary to assume that a chemical union occurs between the two opposing substances. In view of the strict specificity this binding is best explained by assuming the existence of two groups having a definite configuration, of two groups fitting one another like a lock to a key, to use Emil Fischer's apt comparison. Considering the firmness of the union on the one hand, and the fact that neutralization takes place even in very high dilutions without the aid of chemical agents, we must assume that the binding is due to a close chemical relationship, in all probability analogous to a true chemical synthesis.

Recent investigations, in fact, have shown that it is possible, by chemical interference, to disrupt the combination, to split the toxin-antitoxin union into its components. Morgenroth, for example, has shown this with a number of poisons. Thus with snake venom and diphtheria poison he found that the action of hydrochloric acid caused the toxin-antitoxin combination to resolve into its original components, just as in pure chemistry stable combinations such as the glucosides, when acted on by acids, are resolved into their two components, sugar and the constituent aromatic group. These investigations showed that the more stable group of the toxin molecule, the group to which I have given the name "haptophore," is able to exhibit marked chemical activity of specific character, and it was therefore very natural to assume that just this group effected the anchoring of the toxin to the cell. We see, for example, how many species of bacterial poisons take weeks before they produce disturbances, and how they confine their injurious action to heart, kidney, or nerve. We see animals ill of tetanus infection exhibiting spasms and contractures for months. All this compels us to admit that these phenomena can only be caused by the anchoring of the poison by certain definite cell complexes.

I therefore assumed that tetanus poison, for example, united with certain definite chemical groups of the cell protoplasm, particularly of the protoplasm of the motor ganglion cells, and I further believed that this chemical union was the prerequisite and the cause of the disease. These groups I termed "poison receptors," or simply "receptors." Wassermann, through his well-known experiments, was able to demonstrate the correctness of this view, by showing



that normal brain substance is able to neutralize definite quantities of tetanus toxin. A number of objections were made against these experiments, but they proved to carry no weight. I am convinced that it has been proven conclusively that the cells contain definite chemical groups which bind the poison. And that these groups, receptors, react with the haptophore portion of the toxin, is shown by the fact that it is possible to immunize with toxoids, in which, of course, only the haptophore group is present. We know that this haptophore group of the toxins must possess a peculiar, highly complex stereochemical structure, and since it reacts in exactly the same manner both with the antitoxin and with the cell receptors, we conclude that the group contained in the protoplasm, the cell receptor, must be identical with the "antitoxin" present in solution in the serum of the immunized animals. In view of the fact that the cell receptor constitutes the preformed element, while the artificially produced antitoxin represents the result, i. e., the secondary element, it is most natural to believe that the antitoxin is nothing else than thrust-off constituents of the cell, in fact surplus receptors which have been thrust off. The explanation for this is very simple. It is merely necessary to assume that the various specific cell receptors which bind, for example, snake venom, diphtheria poison, tetanus poison, botulism poison, etc., are not intended to serve as *poison catchers* for poisons with which the animal perhaps never comes into contact under ordinary conditions, but that they are really designed to chemically bind *normal metabolic products*, i. e., that they are intended primarily to effect assimilation. These receptors are therefore to be thought of as side chains of the protoplasm possessing the power of assimilation. When laid hold of by a toxin molecule, the particular normal function of this group is lost, put out of action. Thereupon, following the principle discovered by Weigert, the protoplasm not only repairs the injury, but even over-compensates the defect, i. e., there is superregeneration. Finally, with the accumulation and repetition of the injections, so many of these regenerated groups are formed in the body of the cell that they hinder, as it were, the normal cell functions, whereupon the cell rids itself of the burden by thrusting the groups off into the blood.

The most striking thing about this process is the enormous difference between the amount of poison injected and the antitoxin produced. Some idea of this disproportion can be gained from the statement made by Knorr that one part of toxin produces a quantity

of antitoxin sufficient to neutralize one million times the quantity of toxin injected.

There are those, to be sure, who believe the process is much simpler than this. Straub, for example, thinks it is essentially analogous to simple detoxicating phenomena occurring in the body, comparing it, for example, with the formation of an ethereal sulphuric acid from injected phenol. The only difference, Straub believes, is that phenol sulphuric acid is stable in the organism, while the toxin-antitoxin combination is unstable, being partially destroyed in the organism. This destruction, however, affects only one component, the injected toxin, the other, the reaction product of the organism (being related to the organism and therefore not a foreign biological substance) escapes elimination and remains in the blood and body fluids. By systematically repeating the poisoning it is thus possible to increase the protective power of the blood, so that when this blood is injected into other animals the protective power is transformed, and the injected animals become resistant to the toxic infection.

This is Straub's idea. With so simple an explanation, one will wonder why this question has engaged the attention of so many investigators in immunity these many years. As a matter of fact, however, it seems entirely to have escaped the author that according to his theory a certain quantity of toxin can only produce an equivalent amount of antitoxin. Fortunately, however, in immunization this is not the case. It can be shown, as has already been said, that one part of toxin can produce an amount of antitoxin a million times more than the equivalent. This alone is enough to show how untenable Straub's conception is.

Of far greater importance is the fact that the demonstration of this hyperregeneration proves the preformation and the chemical individuality of the corresponding toxin receptors. That which the cell constantly produces and which can be given off to the blood after the manner of a secretion must have a chemical "individuality." This constitutes the first step toward resolving the cell concept into a large number of separate individual functions. From the beginning I had assumed that the toxin represented nothing more than an assimilable food stuff to which in addition, by chance as it were, was attached a side group, very labile in character, which really exerted the toxic action.

This view was very quickly confirmed in a number of ways.

The actual independence of haptophore and toxophore groups was conclusively demonstrated by the discovery of substances which had the power to excite the production of antibodies, and which, therefore, were antigens, without possessing any toxic action. I may remind you of the precipitins first observed by Kraus, Tschis-tovitsch and Bordet. These authors showed that albuminous bodies derived from either animal or vegetable organisms were able to excite the production of specifically reacting antibodies, and this, whether they possessed toxic properties or not. The demonstration of their antigen nature was thus extended to true food stuffs, a result to be expected on the basis of my theory. Moreover, even among the poisons found in nature, some have been encountered in which the independence of the haptophore and of the toxophore apparatus is at once recognized. I refer to cytotoxins which are found normally in the blood serum of certain higher animals, or which can be artificially produced by immunization with any particular species of cell. These cytotoxins differ from all other poisons known to us by the extraordinary specificity of their action by a degree of monotropism possessed, so far as we know, only by the poisons derived from the living animal body. Owing to their complex constitution it is easy to differentiate the haptophore and the toxophore apparatus, and to show that the function of the distributive component, the amboceptor, is to concentrate the really active substance on the affected cell. This is effected by an increase in the affinity of the amboceptor after union with the cell has taken place. The fact that animal cells act as antigens without possessing any toxic action, and the fact that it is possible to immunize with dissolved albuminous substances, demonstrates that only the haptophore group is responsible for the formation of antibodies.

The recognition and the careful analysis of the specific relations existing between the haptophore groups of antibodies and of receptors, has proven of the highest theoretical and practical importance in serum diagnosis. To cite only a few examples, let me call your attention to the determination of the agglutinating titer in its application to the Widal reaction in typhoid fever, to the method of differentiating albumins introduced by Wassermann and Uhlenhuth, and its significance in the forensic diagnosis of blood, to the measurement of the opsonic index introduced by Wright, and to numerous applications which have been made of the method of complement binding, a method whose scientific basis also rests

on the principle of anchoring the antibody to the haptophore group.

Without going further into this subject, I wish merely to emphasize the fact that there are a number of foodstuffs, mostly probably albuminous in character, which find *specific* receptors on the cells, and that we are thus enabled by means of immunization to draw these receptors into the blood. Here they present themselves in various forms as agglutinins, precipitins, amboceptors, and opsonins, and as antitoxins and antiferments. By causing them to accumulate in the blood we can subject these substances to minute analysis, a procedure entirely out of the question so long as they remained part of the cell. The extent to which the analysis of these reactions can be pursued is well illustrated by the study of the toxin-antitoxin combination and by the recognition of the complex character of the amboceptor action.

This, of course, does not by itself solve the mystery of life. Comparing the latter to the complex structure of a mechanical apparatus, we might say that we are at least able to take out some of the wheels and study them minutely. This is certainly a great advance over the former method—to smash the entire apparatus and then hope to learn something from the mass of fragments.

I term all the receptors which are enabled and designed to assimilate foodstuffs for the cell "nutri-receptors." I consider that these nutri-receptors constitute the source of the antibodies mentioned above. From a pluralistic standpoint it is, of course, necessary to assume that there are a large number of nutri-receptors of various kinds. In view of the complexity of the organism, and of the multiplicity and specificity of the cell functions, a standpoint other than this appears out of the question. In immunizing we can distinguish three classes of nutri-receptors, namely:

1. Those which do *not* pass into the blood in the form of antibodies. We may assume that this is the case with nutri-receptors serving the very simplest functions, as, for example, the absorption of simple fats and sugars.

2. Those which pass into the blood in the manner described above, forming characteristic antibodies. The production of these corresponds to a superregeneration.

3. The third form contrasts with the preceding, in that instead of a regeneration, there is a *disappearance* of receptors. Experimental evidence of the occurrence of this form, to be sure, has thus

far been very meagre. The one example which may be familiar to the reader is the fact demonstrated by H. Kossel that on long-continued immunization of rabbits with the hæmotoxic eel serum, the blood cells finally became insusceptible to this serum, as though they had lost their specific receptors.

Recently, aided by my colleagues, Dr. Röhl and Miss Gulbransen, I succeeded in gaining an insight into the nature of the disappearance of receptors. While the work will be made the subject of a special paper, I may here say that our experiments were made on trypanosomes. Working in my laboratory, Franke, after infecting a monkey with a particular species of trypanosome, had cured the disease by means of chemo-therapeutic agents, and had tested the immunity of the animal by again infecting it with the original strain. Contrary to expectations, it was found that the monkey was not immune, so that after a very prolonged incubation, the disease reappeared. If mice were inoculated with blood from the diseased animal, i. e., with blood containing trypanosomes, they became infected and died. Curiously, however, if the trypanosomes were first removed from this monkey blood, it was found that the serum was able to kill the *original* strain of trypanosomes. This showed that the trypanosomes had undergone some change in the body of the monkey, and that the variety thus produced differed from the original strain in its behavior toward the serum; it had become serum-fast. Similar observations were made at the same time by Kleine, and recently also by Mesnil.

We found that when animals which had been infected with a particular strain of trypanosome were treated with less than the complete sterilizing dose of suitable substance (arsanil, arsazetin, arsenophenylglycin) the trypanosomes disappear from the blood for a time. It can easily be shown that in this case also antibody has been produced. The few parasites which escape destruction lie dormant in the body for a time and gradually adapt themselves to the antibodies present in the serum. Then they again pass into the blood, where they rapidly multiply and bring about the death of the animal. We inoculated the trypanosomes so obtained into two series of mice. One series consisted of mice which had been infected with the original strain and then cured with suitable doses. These animals, therefore, possessed specific antibodies. The other series consisted of normal mice. Infection resulted equally rapidly in both series. This shows that the parasites of the strain producing

the relapse have undergone a biological alteration, in that they have become serum-fast.<sup>1</sup> The alteration in these parasites is not superficial in character. On the contrary it may persist for many months and through repeated passage through normal animals. The relapse strain maintains its resistance to the antibodies produced by the original strain, and can thus be positively identified.

It was necessary to attempt to gain an insight into the nature of this alteration. After varying the experiments in many ways we reached the following conclusion: The original strain is plentifully supplied with a certain uniform type of nutri-receptor, which we may term group A. If the parasites are now killed and dissolved in the mouse's body, group A acts as antigen and gives rise to antibodies having definite relationship to group A. When living parasites are brought into contact with this antibody, either in vitro or in vivo, the antibody is anchored by the parasites. As a result of this occupation of its receptors, the parasites undergo the biological alteration which leads to the relapse strain. The alteration consists in the disappearance of the original group A, and its replacement by a new group, B. The following experiment shows that the relapse strain contains a new group. Two mice are infected with the relapse strain, which possesses group B, and are then completely healed. On infecting one mouse with the original strain, the other with the relapse strain, it will be found that infection with the original strain, carrier of group A, is successful, while reinfection with the relapse strain is at first unsuccessful. This shows that the original strain and the relapse strain are not identical, that they must be carriers of two different functional groups. We are dealing, therefore, with a typical case of disappearance of receptors following immunization, and accompanied by the formation of an entirely new variety of receptor.

It is probably of little consequence whether this alteration is regarded as a mutation or a variation. The important thing is that it can be artificially produced at will, and that it is hereditary. In view of the great interest attaching to this problem in biology and embryology, we have attempted a further analysis of the phenomenon.

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<sup>1</sup> Exactly the same strain can be produced in much simpler fashion, by infecting mice with the original strain, and healing the animals on the second day with a full healing dose. After two or three days they are then again infected with the same strain. After a time parasites will appear in the blood, and these will be found to correspond entirely to those of a relapse strain.

To begin, it was necessary to determine in what manner the trypanosome antibodies affected the parasites. Corresponding to our previous knowledge of immunity we could assume that these antibodies exert a direct poisonous action, i. e., that they therefore probably contained toxophore or trypanolytic groups, so that the anchoring of the antibody by the parasite is followed by an injury or even the destruction of the latter. This, however, is not the case. In contrast to the ordinary strains of trypanosomes, which possess only a uniform group, A, B, or C, and which may therefore be termed "Unios," one meets with other strains which possess two groups in their protoplasm, A and B, and which may therefore be termed "Binios." If such a binio "A-B" is acted on by the isolated antibody A or B, growth will not be injured in the least. Not until both antibodies act at once does this occur. From this it follows that the presence of the antibodies does not produce a direct toxic effect on the parasites. To us it seems that this three-fold experiment demonstrates that the antibody acts merely by blocking the food supply by occupying the corresponding receptors. It thus comes to pass that when in the binio A-B the group A is occupied by an antibody, the parasite can continue to vegetate by means of the group B. From this it also follows that groups A and B are essentially nutri-receptors.

If the amount of antibody is very large, the parasite finds it impossible to obtain nourishment, and consequently dies off. This can easily be demonstrated by mixing the parasite in a test tube with varying amounts of antiserum; the parasite is killed in the high concentrations which completely shut off the food supply, while in the weaker concentrations, which permit a *vita minima*, the parasites undergo the alteration already discussed, and give rise to a relapse strain. This mutation is therefore referable entirely to a hunger of the protoplasm, and under this influence the trypanosome develops new potentialities. I have given the name "atrepsins" to antibodies of the type just discussed, i. e., those whose action is purely *antinutritive*, and I believe that they play an important rôle not only with bacteria but in biology in general.

In view of the fact that the presence of antibodies demonstrates the existence of definite chemical groupings, most of the workers in immunity will have no difficulty in accepting the idea that there are definite chemical groups in the cell designed for the taking up of nutritive material. A much more difficult question is as to the

existence of analogous groups for the assimilation of less complex substances. So far as the simplest additional function is concerned, namely, the absorption of oxygen, I believe this question is already partly answered. It is well established that in the hæmoglobin molecule it is exclusively the organically bound iron residue which effects the loose union with the oxygen on the one hand, and the carbon dioxide and hydrocyanic acid on the other. It will therefore be necessary to assume that the red blood corpuscles contain definite groupings which possess a maximum affinity for iron and with that form a complex combination having the characteristic functional properties. The protoplasm of the red blood corpuscles would thus be characterized by a plentiful supply of "ferro-receptors," the completing of which receptors with iron leads to the finished hæmoglobin molecule. Similarly we shall have to assume the existence of "cupri-receptors" in the blue respiratory pigment of crabs, and perhaps of "mangano-receptors" in other animals. The localization of iodine in certain glands, especially in the thyroid gland, and also the fact that the iodine is associated with certain aromatic side chains, will also be interpreted according to this conception.

The question as to whether the cell contains preformed chemo-receptors for the great host of true therapeutic substances is one of great difficulty. This leads us into the important domain governing the relation between chemical constitution and pharmacological action, which in turn constitutes the basis for the rational development of therapeutics. Not until we have really learned the site of attack of the parasites, when we have come to know what I term the therapeutic biology of the parasites, will we wage successful warfare against the producers of infection.

For this reason I have begun studying the existence of particular chemo-receptors on unicellular organisms, because here the conditions are much more favorable for gaining a clear insight than is the case in the extremely complex mechanism of the higher organisms. The problem I undertook to solve was this: Do trypanosomes possess, in their protoplasm, definite groupings which bring about the anchoring of certain particular chemical substances?

If any particular substance possesses the power to kill trypanosomes or other parasites in a test tube or in the animal body, it is obvious that this can only be due to the fact that the substance is taken up by the parasites. This bald fact, however, does not by



self give us an insight into the way in which this is brought about. A large number of different explanations can be brought forward. Not until we can prove that we are dealing with a function which is capable of being altered and varied in a specific manner is it possible to regard the existence of preformed groups as demonstrated.

Unfortunately it seems to be impossible to utilize the method employed in demonstrating the preformed existence of nutri-receptors, namely, by causing the liberated receptors to be thrust off to the blood. The chemo-receptors appear to be much more simply constituted, and remain attached to the cell, so that no rusting-off occurs.

By indirect means, however, we succeeded in getting light on this phase of the subject. With the aid of my esteemed collaborators, Franke, Browning and Röhl, I was able to show that it is possible, by systematic treatment, to produce strains of trypanosomes possessing immunity against the three trypanocidal poisons now known to us. These poisons, it will be remembered are (a) substances of the arsenic group, (b) fuchsin, and (c) the acid dye known as trypanred belonging to the benzoburpurin series. The immune strains are marked by two characteristics:

1. A stability of the acquired character. This is very great. Thus our arsenic strain, after having been passed some 380 times through mice in the course of two and one-half years, still possesses the same drug immunity as the original strain.

2. An essential feature of the immunity to drugs is the strict specificity. This manifests itself by the fact that the immunity is related not against a certain definite elementary combination, but against the entire chemical group of which this combination is a part. Thus the strain made immune against fuchsin is resistant not only to that substance but also against a large number of related diphenylmethane dyes, e. g., malachite green, ethyl green, hexæyl violet. In contrast to this, however, the strain has remained susceptible to the action of the two other types, i. e., against trypanred and against an arsenical. A corresponding specific resistance exhibited by the strain made fast against trypanred and by the one made fast against arsenic preparations. That we are here dealing with three different functions is further shown by the fact that by successive treatment of a given strain with the three substances mentioned above we can produce a strain which is resistant against all three classes of substances, i. e., one which is triple fast.

Provided that the resistance thus produced is of maximum intensity, such a strain is extremely useful in identifying new types of trypanocidal agents. If, for example, a new substance is encountered which is able to kill ordinary trypanosomes, we have merely to test its action on this triple-fast strain in order to determine whether the substance really represents a new type of trypanocidal agent. If it does not, we shall find that treatment with this substance does not cause the parasites to disappear; on the contrary they multiply. If they disappear, however, we can conclude that the substance does not correspond to any of the three types mentioned, but represents a new type of trypanocidal agent. The triple-fast strain thus acts as a kind of *cribrum therapeuticum*, by the aid of which it is possible to recognize substances belonging together and to separate unrelated substances.

It was now necessary to determine in what manner this specific drug resistance is brought about, and for this purpose I undertook a series of experiments with the atoxyl strain. In order to gain a clear insight into the question it seemed advisable to study the behavior of the arsenic-fast strains, also in a test tube, away from all disturbances and complications of the animal organism. This method very soon encountered a great obstacle, for it was found that the drug mostly used therapeutically, namely, atoxyl (paramidophenylarsinic acid), does not exert the least destructive action on trypanosomes *in test-tube experiments*. Even solutions containing several per cent. of the substance proved insufficient for this purpose. This phenomenon was all the more remarkable because in the human body, according to Koch, injections of 0.5 g. atoxyl suffice to cause the disappearance of the parasites within a few hours. In this case, therefore, destruction is effected in a concentration of 1 to 120,000.

We are here dealing with a phenomenon which is usually spoken of as "indirect action." It was not difficult for me to discover the reason for this peculiar behavior, as I had for years busied myself with reducing power of the animal organism. We know that in the body arsenic acid is transformed into arsenious acid; that cacodylic acid is reduced to the ill-smelling cacodyl. It was natural, therefore, to think first of reductions. In atoxyl, paramidophenylarsinic acid, the arsenic is pentavalent, whereas in the two products obtained from atoxyl by reduction the arsenic is trivalent. In this way we obtained two different products: 1. The monomo-

lecular *p*-aminophenylarsenoxid and 2. The further product, obtained from the latter by reduction, the yellow diamidoarsenobenzol.

In contrast to atoxyl, these substances exhibited marked trypanocidal properties not only in the animal body but also in the test tube. Thus a solution of the arsenoxid combination of a strength of 1 to 1,000,000 killed the trypanosomes in an hour. The closely related *p*-oxyphenylarsenoxid was still stronger killing in 1 to 10,000,000.

This proved that the pentavalent arsenic residue possesses no trypanocidal properties whatever; these are bound exclusively to the trivalent, unsaturated form.

As long as sixty years ago, Bunsen, with extraordinary insight, pointed out that cacodyl, the reduction product, is extremely poisonous, while cacodylic acid is almost non-toxic. This gave him the clue to the chemical character of the cacodyl combination. In striking agreement with this is the fact that the unsaturated carbon oxid, for example, and a number of other unsaturated combinations are so much more toxic that the corresponding saturated combinations. We shall, therefore, have to assume that the arseno-receptor of the cells is able to take up only the unsaturated arsenic residue, i.e., the group possessing the greater combining affinity.

With the aid of such reduced combinations it was simply a matter to test the atoxyl strain in test-tube experiments. These showed that the organisms could be killed with a suitable concentration of the chemical substances, and that we were not dealing with a loss of receptors as in the case of the relapse strain. A comparison, however, of the lethal dose with the dose sufficient to kill the ordinary strain, showed that the resistant strain required a much higher concentration. Amounts which effected immediate destruction of the ordinary strain did not in the least affect the vitality of the resistant parasites, even after one hour.

These test tube experiments seemed to indicate that the arseno-receptor, while still preserved in the atoxyl-fast strain, had undergone some modification so that its affinity had become lessened. This manifests itself by the fact that it required much stronger solutions to produce the poison concentration necessary to effect destruction of the parasites; the normal arseno-receptor of the original strain, by virtue of its higher affinity, takes up the same amount even from more dilute solutions.

We have succeeded in clearly demonstrating by biological methods

that the arseno-receptor actually represents a distinct function whose affinity can be systematically decreased step by step by immunization. Thus far we have obtained three degrees of affinity. (Grade I was produced by subjecting the parasites systematically to the action of *p*-amidophenylarsinic acid and its acetyl combination. We carried out this treatment *ad maximum* for years, until finally no further increase in resistance was produced. The resistant strain thus obtained proved to be resistant at the same time to a number of other arsenicals, among them particularly, the *p*-oxycombination, the combination with urea, and with benzyliden, and a number of acid derivatives.

In practical therapeutics in man and animals, it is, of course, possible that arsenic-fast strains develop; and these, naturally, will absolutely hinder therapeutic success. In animal experiments this is a common occurrence. In view of this it is important to discover substances able still to attack these resistant strains, substances able to combine with their receptors. After long search we found altogether three combinations, of which the most important is arsenophenylglycin. With the aid of this combination it is possible to heal infections produced by the arsenic-fast strain I, which was described above. This can only be explained by assuming that the arsenophenylglycin lays hold on what is left of the arseno-receptor, somewhat as a stump is grasped by a pair of pliers. The anchoring of this substance, however, furnishes a possibility for still further increasing the arsenic-resistance of the strain. After considerable effort we succeeded in producing, out of arsenic strain I, a more resistant strain, arsenic strain II, which was entirely unaffected by arsenophenylglycin.

Plimmer has recently called attention to tartar emetic as a substance which kills trypanosomes, even in high dilutions. Tartar emetic is the salt of an antimony combination, and antimony, it is well known, is closely related to arsenic. On testing arsenic strain II with tartar emetic, we found that the parasites were destroyed by the tartar emetic. By treating arsenic strain II with arsenious acid we were able to produce a still further increase in resistance, so that arsenic strain III was resistant even against tartar emetic. I want to call particular attention to the fact that this arsenic strain III, produced only under the influence of arsenious acid, was resistant to tartar emetic *but not against arsenious acid*. This can only be explained by assuming that of all conceivable arsenicals, arsenious acid is the one possessing the greatest affinity to the arsenic receptor,

and that only by the greatest effort, if at all, will it be possible to produce a strain (which would be arsenic strain IV) resistant also against arsenious acid.

I can adduce many other interesting facts to support my view that under the influence and attack of selected combinations, there is a successive decrease in the affinity of the receptor for that combination. Thus, we have found that we can at once employ one of the stronger agents producing resistant strains, using, for example, arsenophenylglycin. Corresponding entirely to our expectations, the strain thus produced proved resistant also against the less powerful substances, such as atoxyl, arsacetin, etc. A pan-resistant strain would, therefore, be obtained if from the outset we employed the most powerful agents, namely, tartar emetic and arsenious acid. Unfortunately, it appears from our work that it is impossible, at least in small laboratory animals, to directly use these substances for this purpose: it is necessary to proceed indirectly, by treating the organisms first with phenylarsinic acid derivatives.

The loss of affinity is, of course, a chemical phenomenon, and evidently to be interpreted by assuming that in the neighborhood of the arsenic receptor group other groups arise or disappear and thus cause the affinity to be reduced. The following chemical example will serve to illustrate the point. Benzylecyanid reacts with nitrosodimethylanilin. In order that the reaction take place, however, heat and a strong condensing agent, free alkali, are required. However, on introducing a nitro group into the benzole nucleus, the reactivity of the methylen group is markedly increased, so that the two substances, nitro-benzylecyanid and nitrosodimethylanilin, react even in the cold. In this case, therefore, the introduction of the nitro group has exercised a quickening influence on the reaction. If, however, the nitro combination is reduced to *p*-amidobenzylecyanid, we find that the latter is less active than the original material. The amido group has suffered a reduction of affinity. The acetyl product of the amido combination, on the other hand, reacts to about the same degree as the original material.

This simple illustration shows that three different groups attaching to the benzole nucleus in the para position either *increase* the affinity of the methylen group, or *decrease* it, or leave it *unchanged*. The reduction of affinity here observed would correspond to the affinity which we have described above.

According to my view, then, we should consider protoplasm as

made up of a large number of individual functions, which, in the form of different chemo-receptors are scattered amongst the nutri-receptors. I believe that these two main groups cannot but be closely related, and for the following reason.

Trypanosomes of different origin, as they are cultivated in different laboratories, usually from the outset behave differently toward a particular therapeutic substance. The first strain of trypanosome with which I worked, Mal de Caderas, had no resistance whatever against trypan red, and this substance could be employed to effect a cure. This still holds true. Similar favorable results were obtained by Jakimoff in Russia, while Uhlenhuth obtained absolutely no result with this substance on the strains which he used. We are therefore dealing with natural differences in the various strains. Despite the fact that my strain has now been passed through normal mice for many years, it can still be cured by trypan red just as well as ever. This shows that the difference is not entirely artificial. On the other hand, my Nagana strain could formerly not be healed by trypan red, and cannot be healed by that substance now. However, on transforming this Nagana strain into a relapse strain, we were surprised to find that this property, which had persisted for many years, become altered within 14 days. This proves that the chemo-receptors really are related to the constitution of the protoplasm, and undergo alterations when we alter the constitution of the protoplasm.

Whether the reverse holds true, that is, whether, by influencing the chemo-receptors we can alter the cell substance, particularly the nutri-receptors, has not yet been definitely decided. Browning, to be sure, has observed that by means of serum reactions one can differentiate the fuchsin strain from the atoxyl strain, and both from the original strain. Careful investigation subsequently showed, however, that the changes in question were not specific alterations related to the fuchsin or to the arsenic, but alterations which correspond to the relapse mutation described above. These are due to the fact that during the treatment it often happens that the mice suffer relapses, which in turn lead to the formation of relapse strains.

This brings me to the close of my paper. I am well aware that what I have offered you has been quite fragmentary, but this could hardly be otherwise, for the adequate discussion of this theme would mean the recapitulation of an almost endless amount of work. My object in presenting this subject has been to show you that we are

gradually approaching the problem of securing an insight into the nature of the action of drugs. I hope, too, that a systematic application of the views I have here presented will facilitate a rational development of the science of drug synthesis. In this connection I may say that thus far arsenophenylglycin has proven in animal experiments to be a truly ideal therapeutic agent. By the aid of this substance it is possible to completely cure every kind of trypanosome infection in any kind of animal, and that by means of but a single injection. Truly, such a result may be termed *therapia sterilisans magna*.

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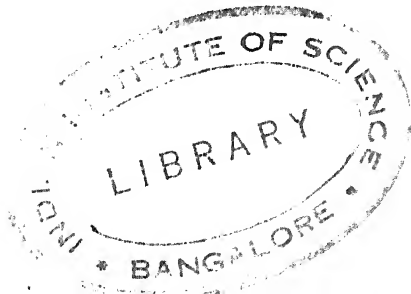


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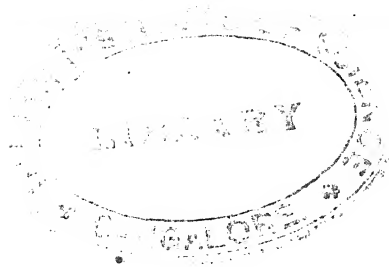
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